

Instrumental Food Analysis

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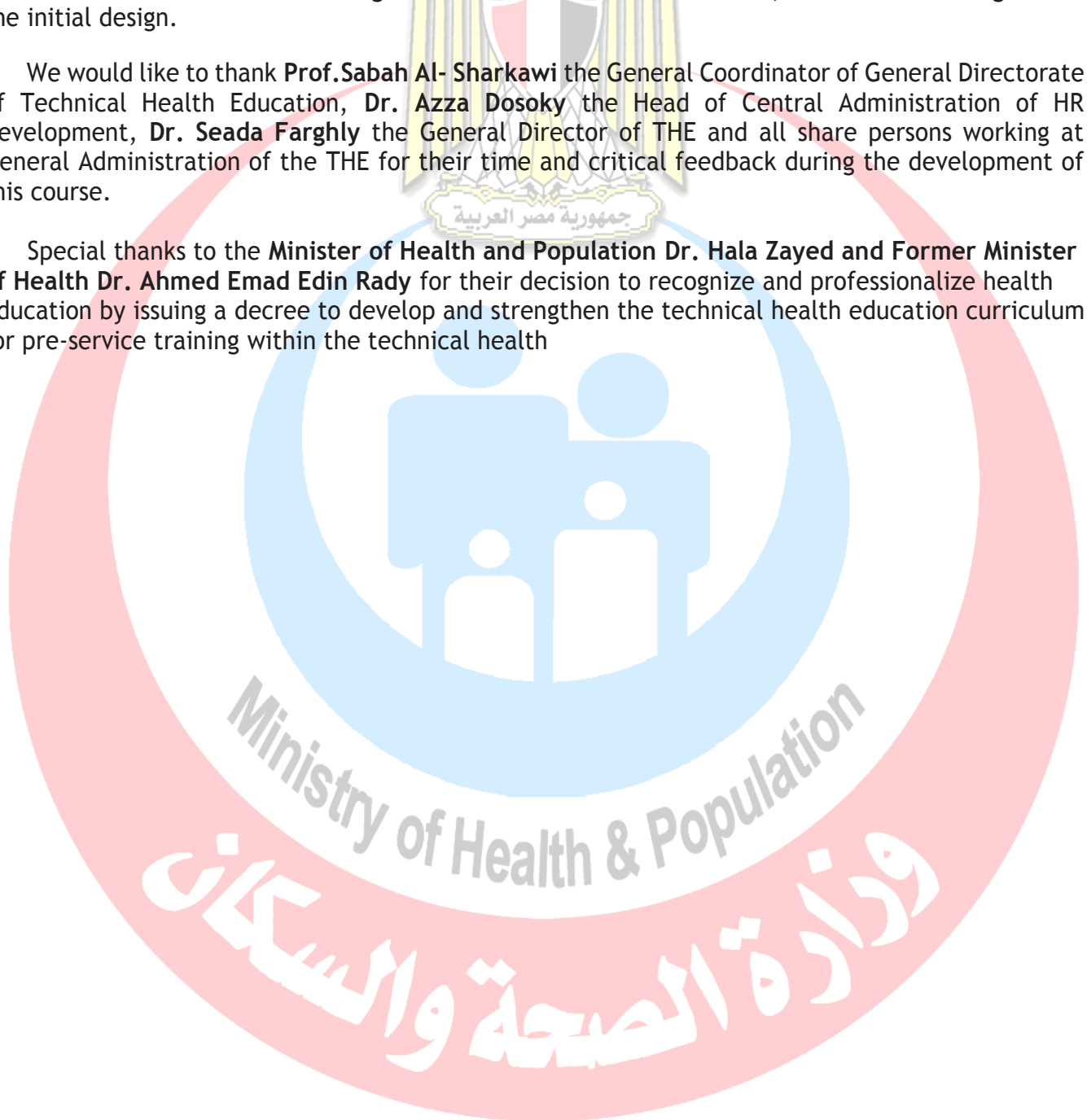


Acknowledgments

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توصيف مقرر دراسي

1- بيانات المقرر		
الرمز الكودي:	اسم المقرر:	الفرقة / المستوى
	Instrumental Food Analysis أجهزة تحليل الغذاء	الثانية
التخصص:	عدد الوحدات الدراسية : نظري	عملي
تغذية علاجية	2	5

2- هدف المقرر:

The course aims to provide knowledge and practical application on the subject of food analysis as well as the relationship between food analysis and food quality. Students will learn about use food analysis in monitoring performance characteristics of food processing steps. . They also will be able to calibrate analytical instruments and interpret the results obtained. This course covers different types of modern analytical instruments such as HPLC, GC, Spectrophotometer, Coupling HPLC to a mass spectrometer, Ultra-High Performance Liquid Chromatography (UHPLC) as well as methods of titration.

3- المستهدف من تدريس المقرر :

1. Recognize the principles of a variety of analytical and instrumental techniques and methods used for measuring the chemical and physical properties of food.
2. Identify Instruments adjustments, accuracy & precision assessments
3. Identify principles of analytical methods photometry, colorimetry, gravimetry, refractometry and chromatographic methods for food analysis.
4. Recognizes principles of protein, fat, carbohydrate and minerals analysis of foods using instruments.
5. Identify protein fractions of foods using Gel Electrophoresis.

أ. المعلومات والمفاهيم :

6. Recognize spectroscopy instruments theory and applications in the food industry.
7. Recognize microwave-assisted process and its application in food analysis.
8. Recognize theoretical determination of vitamins in foods using HPLC.
9. Describe the determination of toxic constituents in foods using modern instrument techniques.
10. Identify the physical properties, including rheological (viscometer) and mechanical properties (texture analyzer) of foods.
11. Identify laboratory safety measures during experimental procedures.

- Interpret and presenting analytical data including use of common calculations, and resources relevant to food analysis.
- Evaluate results obtained from the quantitative and qualitative analysis of food and recognized their indication (Data analysis Techniques)

ب- المهارات الذهنية :

1. Design experimental food analysis for compositional and nutritional quality evaluation.
2. Conducts solutions preparations and calculations.
3. Integrate sampling and treatment of food samples for proximate analysis.
4. Identify proper selection and application of appropriate methods of analysis.
5. Apply statistically valid sampling techniques to food materials having widely diverse properties and volumes.

ج- المهارات المهنية
الخاصة بالمقرر:

6. Demonstrate competency in the use of standard techniques of food analysis and the treatment of experimental data. 7. Calculate and identification of “unknown” compounds using standard curves . 8. Identify the statistical analysis of result reports of food samples. 9. Use food analysis in monitoring performance characteristics of food processing steps.	
- Identify the ethical principles of food analysis. - Adjust instruments and accuracy evaluation. - Learn how to work cooperatively with a laboratory team. - Learn how to behave in Emergency cases in the labs.	د- المهارات العامة:

Online Learning Teaching materials and course documentation will be posted on the institute website. From time to time information about assignments and Practical's are disseminated to students via blackboard. Lecture PowerPoint files will be available on request via blackboard. Learning & Teaching Modes Lectures supported by practical to develop the material covered in the lectures. Time allocated to lectures and practical's can be used for tutorials on request.		4- أساليب التعليم والتعلم
Not applicable		5- أساليب التعليم والتعلم للطلاب ذوي القدرات المحدودة
		6- تقويم الطلاب :
Student Assessment Methods	Assessment Schedule	Weighting of Assessments
Written exam	Determined by Institute administration	60%
Quiz's	Week no. 5 and 11	10%
Practical exam	Week no. 14	30%

7- قائمة الكتب الدراسية والمراجع :

أ- مذكرات

ب- كتب ملزمة

ج- كتب مقترحة

د- دوريات علمية أو
نشرات الخ

Nielsen, S. (2010). Food Analysis Laboratory Manual. 2nd Ed., Springer New York Dordrecht Heidelberg London.

Nielsen, S. (2017). Food Analysis. 5th Ed., Springer International Publishing.

Otles, S. (2009). Handbook of Food Analysis Instruments. CRC Press, Taylor and Francis Group.

Course Description

The course aims to provide theoretical information and practical applications on food analysis to determine the composition and quality of food products. Students will learn about use food analysis in monitoring performance characteristics of food processing steps. They also will be able to calibrate analytical instruments and interpret the results obtained. This course covers different types of modern analytical instruments such as HPLC, GC, Spectrophotometer, Coupling HPLC to a mass spectrometer, Ultra-High Performance Liquid Chromatography (UHPLC) as well as methods of titration. The course also includes instruments for estimating the physical properties of foods such as viscosity and textures.

Core Knowledge

By the end of this course, students should be able to:

- Recognize the principles of a variety of analytical and instrumental techniques and methods used for measuring the chemical and physical properties of food.
- Identify Instruments adjustments, accuracy & precision assessments.
- Identify principles of analytical methods of food analysis.
- Recognizes principles of moisture, protein, fat, carbohydrate and minerals analysis of foods using instruments.
- Identify of protein fractions of foods using Electrophoresis.
- Recognize spectroscopy instruments and applications in the food industry.
- Recognize theoretical determination of vitamins in foods using HPLC.
- Identify the physical properties, including rheological (viscometer) and mechanical properties (texture analyzer) of food.
- Identify laboratory safety measurers during experimental procedures.

Core Skills

By the end of this course, students should be able to:

- Design experimental food analysis for compositional and nutritional quality evaluation.
- Integrate sampling and treatment of food samples for proximate analysis.

- Identify proper selection and application of appropriate methods of analysis.
- Demonstrate competency in the use of standard techniques of food analysis and the treatment of experimental data.
- Identify the statistical analysis of result reports of food samples.

Course Overview

ID	Topics	Methods of Teaching/Training with Number of Total Hours per Topic				
		Interactive Lecture	Field Work	Class Assignments	Research	Lab
1	Introduction to Food Analysis	2				5
2	Assessment of Analytical Methods and Data	2		1		4
3	Sampling and Sample Preparation	2				5
4	Physicochemical Properties of Foods	4				5
5	Compositional Analysis of Foods	6				15
6	Gel Electrophoresis in Food Analysis	2				10
7	Spectroscopy Instruments and Applications in Food Analysis	4				10
8	Chromatography Principles and Applications in Food Analysis	4				10
TOTAL HOURS (91.0)		26				65

Chapter 1

Introduction to Food Analysis

Objectives

- After reading this chapter, you should be able to:
 - Explain the safety precautions in food analysis laboratories.
 - Define steps of food analysis.

Food Analysis Lab.

What is Food analysis laboratory?

- The food analysis laboratory relies on three **main points**: analyst, the method of analysis and instruments in a harmonious system to perform the analysis (**Figure 1.1**).

Analyst Responsibilities:

As an analytical chemist, your tasks can vary, but you will **typically need to**:

- Apply safety requirements when conducting the experiment.
- Analyze samples from various sources to provide information on compounds or quantities of compounds present.
- Use analytical techniques and instrumentation, such as gas and high performance liquid chromatography (HPLC), and spectroscopy.
- interpret data and meet strict guidelines on documentation when recording data.
- report scientific results.
- work collaboratively in cross-functional teams.
- Identify process of instruments calibration.

Method of analysis:

- The choice of method for a specific characteristic or component of a food sample is often made easier by the availability of official methods.

- Analysis of Association of Official Agricultural Chemists (**AOAC**) is an international source of methods, in which scientists worldwide contribute their expertise to standards development, method development, and the systematic evaluation and review of methods. It is the most comprehensive collection of chemical and microbiological methods available in the world, and many methods within the compendium have notation indicating their adoption as harmonized international reference methods by the International Organization for Standardization (**ISO**), the International Dairy Federation (**IDF**), the International Union of Pure and Applied Chemistry (**IUPAC**), and the **Codex Alimentarius Commission**.

Instruments considerations:

- Equipment used for food analysis should be periodically calibrated; Instrument calibration is one of the primary processes used to maintain instrument accuracy. Calibration is the process of configuring an instrument to provide a result for a sample within an acceptable range. Eliminating or minimizing factors that cause inaccurate measurements is a fundamental aspect of instrumentation design.
- The goal of calibration is to minimize any measurement **uncertainty** by ensuring the accuracy of test equipment. Calibration quantifies and controls errors or uncertainties within measurement processes to an acceptable level.

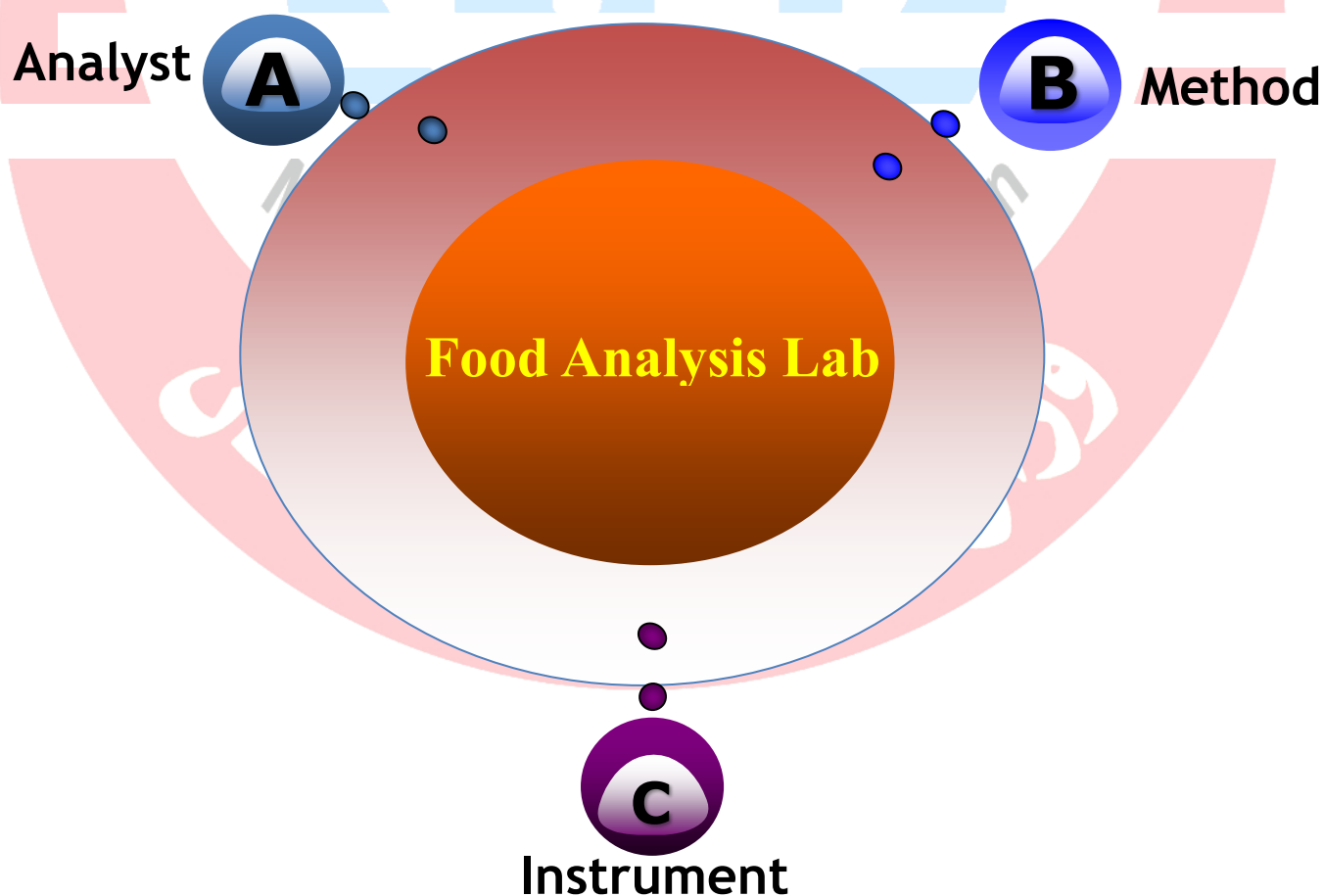


Fig. 1.1. Basics of Food Analysis Lab

General Laboratory Safety Rules:

Before Lab Work, Get to Know:

- Hazards of materials & agents and their prescribed safety procedures.
- Emergency spill procedures, use of adsorbents and disinfectants.
- Designated escape route and alternate emergency exits.
- Location of fire extinguisher, emergency eyewash and safety shower.

While Working in the Lab:

- Restrain long hair, loose clothing and jewelry.
- Use appropriate eye, skin, and hand protection (e.g. lab coats, safety glasses, gloves).
- No food, beverages, tobacco products, or application of cosmetics in the lab.
- Handle unknown materials as if they were hazardous.
- Handle volatiles in a fume hood.
- Use mechanical transfer devices (e.g. pipetting aids; no mouth pipetting).
- Label and store all chemicals appropriately.
- Minimize clutter, keep lab and bench tidy, and dispose of waste regularly.
- Report all accidents, injuries, fires, spills to take appropriate corrective actions.

Before Leaving the Lab:

- Turn off:
 - Gas
 - Water
 - Power supplies
 - Vacuum lines
 - Compression lines
 - Heating apparatus
- If necessary, identify and package waste, dispose properly.
- Lock/out and tag/out defective equipment.
- Decontaminate work surfaces and equipment.
- Leave lab coat and gloves in the lab.
- Wash hands.
- Close and lock door.



Regular Lab Safety Checks:

- Acids and solvents must be stored segregated in storage space under fume hood or solvent cabinets. Do not store them on the working surface of the fume hood.
- Store flammables in approved safety cans or cabinets, segregate from oxidizing acids and oxidizers.
- Purge emergency eye wash (weekly, document on sign up sheet next to the eyewash).
- Check accessibility of emergency showers and eyewashes.
- Check that first aid kit is complete.
- Check fume hood for proper operation.
- Check tubing, pressurized connections, gas.
- Check chemical storage.
- Regularly review the types of hazards in the lab (chemical, physical, biological, ergonomic, mechanical)

Chemical Hazard Symbols and Definitions:

Before you start handling of chemicals, look at the label on the bottle you will find one of the following: (Figure 1.2)

• Flammable



• Irritant



• Explosive



• Corrosive



• Toxic/Poison



• Environmental



Fig. 1.2. Chemical Hazard Symbols

- **Flammable** - Any substance that will burn if exposed to an open flame.
- **Explosive** - A substance that may explode if exposed to heat or flame.
- **Toxic/Poison** - A substance that can lead to death if inhaled, ingested, or absorbed by the skin.

- **Corrosive** - A substance that can destroy or burn living tissue and can eat away at other materials.
- **Irritant** - A substance that causes inflammation upon contact with skin or mucous membranes.
- **Environmental** - Substances that are harmful to the environment. They must be disposed of properly, not washed down the drain

Lab Safety Equipment

The safety procedures in the food analysis laboratory are based on the use of and safety tools while ensuring the validity of these tools continuously (Figure 1.3)



Fig. 1.3. Lab Safety Equipment

Why food analysis is important?

Food analysis is important to **monitor food composition** and to ensure the **quality and safety** of the food supply. All food products require analysis as part of a quality management program throughout the development process (including raw ingredients), through production, and after a product is in the market. The characteristics of foods (i.e., chemical composition, physical properties, sensory properties) are used to answer specific questions for regulatory purposes and typical quality control. The nature of the sample and the specific reason for the analysis commonly dictate the choice of analytical methods.

▪ Types of sample analyzed

Chemical analysis of foods is an important part of a **quality assurance program** in food processing, from ingredients and raw materials, through processing, to the finished products. Chemical analysis also is important in **formulating and developing new products**, evaluating new processes for making food products, and identifying the source of problems with unacceptable products. For each type of product to be analyzed, it may be necessary to determine either just one or many components. The nature of the sample and the way in which the information obtained will be used may dictate the specific method of analysis. For example, process control samples are usually analyzed by **rapid methods**, whereas nutritive value information for nutrition labeling generally requires the use of more time-consuming methods of analysis endorsed by **scientific organizations**.

Steps of Food Analysis

- A. Select and prepare sample.
- B. Perform the assay.
- C. Calculate and interpret the results.

Chapter 2

Assessment of Analytical Methods and Data

Objectives

- After reading this chapter, you should be able to:
 - Identify the proper selection and application of appropriate methods of analysis.
 - Interpret and presenting analytical data including use of common calculations, and resources relevant to food analysis.
 - Evaluate results obtained from the quantitative and qualitative analysis of food and recognized their indication.

Requirements and choice of analytical methods

The choice of method(s) used for the analysis of foods is dependent on a number of factors and relates to the following features. Their desirability or otherwise needs to be considered in deciding on a particular analytical procedure.

Precision: a measure of the ability to reproduce an answer between determinations performed by the same scientist or by different scientists in the same laboratory using the same procedure and instrument(s). In other meaning, precision is the amount of scattering in replicate measurements of the same quantity and is expressed in terms of deviation. Measurement, sampling, and calibration errors all contribute to decreased precision and increased uncertainty.

Reproducibility: similar in principle to precision but based on the ability to reproduce an answer by different analysts and/or laboratories using the same procedure.

Accuracy: expressed in terms of the ability to measure what is intended to be measured, e.g. the fat content of a foodstuff rather than all substances of similar solubilities, or the protein content of a food rather than all nitrogen containing substances. In another meaning, the difference between a measured value and the true value is expressed in terms of error. A consistent error, such as one caused by an improperly prepared reagent, may produce replicate results that are similar but inaccurate by the same amount. This type of error is known as bias. For example, in the moisture analysis for processed cheese spread the result obtained was 45.25

%. Let us say the true moisture value was actually 45.51%. By comparing these two numbers, you could probably make a guess that your results were fairly accurate because they were close to the correct.

Economy: expressed in terms of the costs involved in the analysis in terms of reagents, instrumentation and time.

Speed: based on the time to complete a particular analysis. This could be important, for example, where any necessary follow-up action needs to be undertaken quickly, e.g. the recall of food products containing higher or lower levels than the permissible amounts of a particular constituent.

Sensitivity: measured in terms of the capacity of the method to detect and quantify food constituents and/or contaminants at very low concentrations such as might occur with trace elements or pesticide residues. Modern methods of food analysis such as gas chromatography enable detections to be obtained at levels as low as 10^{-10} g, while more established colorimetric methods are sensitive to levels of around 10^{-7} g. Traditional methods of gravimetric and titrimetric analysis, on the other hand, may only allow measurements to levels of around 10^{-3} g.

Specificity: expressed in terms of the ability to detect and quantify specific food constituents even in the presence of similar compounds, e.g. the estimation of individual sugars present in a food containing a range of both reducing and non-reducing sugars.

Safety: many reagents used in food analysis are potentially hazardous; hazards include the corrosiveness of acids and the flammability of some organic solvents.

Official approval: various international bodies give official approval to methods that have been comprehensively studied by independent analysts and shown to be acceptable to the various organizations involved. These include:

- ISO (International Organization for Standardization)
- AOAC (Association of Official Analytical Chemists; published in the AOAC methods book)

The eventual choice of a method will thus depend on which of the above factors is most critical. In matters of dispute or involving **legislative requirements**, the use of an **officially approved method** could be of utmost importance, while for the purposes of routine analysis for quality control, speed, cost, and precision could have a more important bearing.

Quality of data:

All measurements are subjected to various degrees of error which may be either quality of data inherent in the equipment or procedure, and are thereby difficult to avoid, or may be the result of poor technique or design and can be reduced or eliminated by undertaking various procedural steps to avoid such errors.

Systematic or determinate errors are usually errors of procedure peculiar to each particular method and may not normally be treated statistically. They include factors such as problems with the design or age of instruments and errors attributable to the presence of interfering compounds in a food mixture, e.g. the estimation of reducing sugars may be affected by the presence of other, non-carbohydrate, reducing compounds in the mixture. Another example, a pipette that consistently delivers the wrong volume of reagent will produce a high degree of precision yet inaccurate results. Sometimes impure chemicals or the analytical method itself are the cause. Generally, we can overcome systematic errors by proper calibration of instruments, running **blank** determinations, or using a different analytical method.

Random errors may arise from a number of sources but may be minimized by using **replicates** and calculating **means**. They include human errors arising from the incorrect reading of such equipment as pipettes, burettes, judging the endpoint change in a titration, and instruments possessing analog rather than digital scales, and may also arise from badly designed experiments where excess light or temperature may cause decomposition of a food ingredient such as ascorbic acid.

✚ Procedures to improve quality of data:

A number of steps and precautions may be undertaken to avoid many of the errors indicated above and to improve the reliability of data produced. These include the following:

- **Quality of glassware:** The glassware (pipettes, burettes, volumetric flasks, cuvettes, etc.) and equipment being used for the analysis should be of a quality appropriate to the degree of precision required.
- **Handling equipment:** Glassware and equipment should be handled in the correct manner; for example, volumetric flasks, which are calibrated to specified temperatures, should not be heated.
- **Blank analyses:** In order to ensure that background interferences from materials used in an analysis are not occurring, the analysis of a reagent blank should be carried out wherever possible. **This blank should contain all the reagents used in the test sample but exclude the sample itself.** Values obtained in the blank analysis should then be **subtracted** from those obtained with the sample being analyzed.

- **Replication:** As many replicates as possible should be performed in order to **minimize** the effects of **random error**.
- **Reference samples:** The validity of an analytical procedure may be estimated by carrying out analyses on foods of known composition. Such standard food samples are available commercially and are an invaluable measure of the effectiveness of methods such as in the estimation of dietary fiber.
- **Collaborative testing:** By collaboration with a number of laboratories, the results obtained by a particular laboratory may be compared with those being achieved by others using the same method. This allows the detection of any routine errors within anyone laboratory where the results are consistently different from those of other participants in the scheme.
- **Confirmatory analysis:** The results obtained by any particular method being used should be compared against those obtained by a reference method chosen from one recognized internationally and published by bodies such as **ISO** and **AOAC**. This allows a measure to be made of the **validity** of the method being used for routine purposes.

Statistical assessment of quality of data

To increase accuracy and precision, as well as to evaluate these parameters, the analysis of a sample is usually performed (repeated) several times. At least three assays are typically performed, though often the number can be much higher. Because we are not sure which value is closest to the true value, we determine the mean (or average) using all the values obtained and report the results of the mean. The mean is designated by the symbol $\bar{x}$ and calculated according to the equation [2.1]

$$\bar{x} = \frac{x_1 + x_2 + x_3 + \dots + x_n}{n} = \frac{\sum x_i}{n} \quad \text{Eq. 2.1}$$

Where:

$\bar{x}$ = mean

x_1, x_2 , etc. = individually measured values (x_i)

n = number of measurements

For example, suppose we measured a sample of processed cheese spread for percent moisture content four times and obtained the following results: 45.22%, 45.45%, 45.10%, and 44.91%.

$$\bar{x} = \frac{45.22 + 45.45 + 45.10 + 44.91}{4} = 45.17\%$$

Accuracy and precision

The problem in determining **accuracy** is that most of the time we are not sure what the true value is. For certain types of materials, we can purchase known samples from, for example, the National Institute of Standards and Technology and check our assays against these samples. Only then can we have an indication of the accuracy of the testing procedures. Another approach is to compare our results with those of other labs to determine how well they agree, assuming the other labs are accurate.

A term that is much easier to deal with and determine is **precision**. This parameter is a measure of how reproducible or how close replicate measurements become. If repetitive testing yields similar results, then we would say that the precision of that test was good. From a true statistical view, the precision often is called error, when we are actually looking at experimental variation. So, the concepts of precision, error, and variation are closely related. The difference between precision and accuracy can be illustrated best in **Figure 2.1**. Imagine shooting a rifle at a target that represents experimental values. The bull's eye would be the true value and where the bullets hit would represent the individual experimental values. As you can see in **Figure 2.1 a**, the values can be tightly spaced (good precision) and close to the bull's eye (good accuracy), or, in some cases, there can be situations with good precision but poor accuracy (**Figure 2.1 b**). The worst situation, as illustrated in **Figure 2.1 d** is when both the accuracy and precision are poor. In this case, because of errors or variation in the determination, interpretation of the results becomes very difficult.

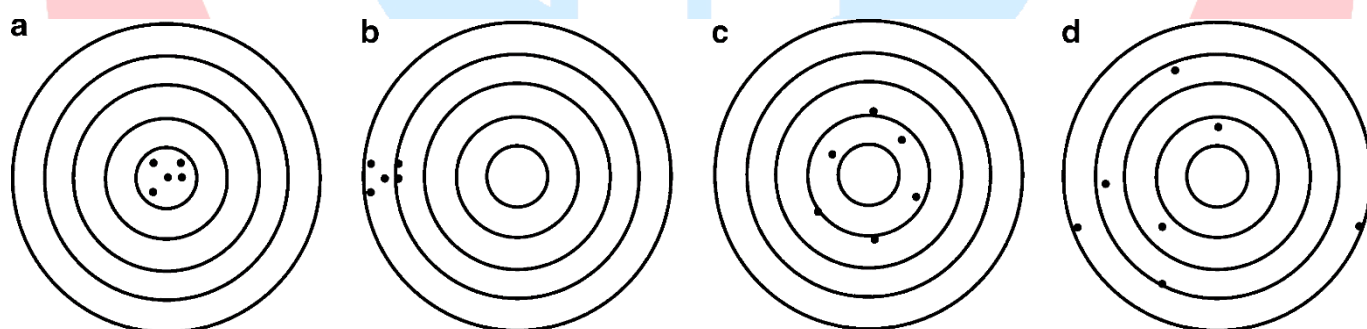
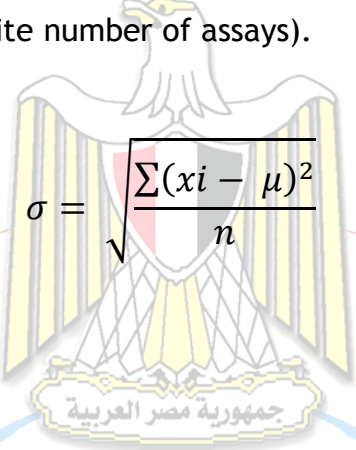


Fig. 2.1 Comparison of accuracy and precision: (a) Good accuracy and good precision, (b) Good precision and poor accuracy, (c) Good accuracy and poor precision, and (d) Poor accuracy and poor precision.

Probably the best and most commonly used statistical evaluation of the precision of analytical data is the **standard deviation**. The standard deviation measures the spread of the experimental values and gives a good indication of how close the values are to each other. When evaluating the standard deviation, one has to remember that we are never able to analyze

the entire food product. That would be difficult, if not impossible, and very time-consuming. Thus, the calculations we use are only estimates of the unknown true value.

If we have many samples, then the standard deviation is designated by the Greek letter sigma (σ). It is calculated according to **Equation [2.2]**, assuming all of the food products were evaluated (which would be an infinite number of assays).



$$\sigma = \sqrt{\frac{\sum (xi - \mu)^2}{n}}$$

Eq. 2.2

Where:

σ = standard deviation

xi = individual sample values

μ = true mean

n = total population of samples

Because we do not know the value for the true mean, the equation becomes somewhat simplified so that we can use it with real data. In this case, we now call the σ term the standard deviation of the sample and designate it by SD or σ . It is determined according to the calculation in **Equation [2.3]**, where $\bar{x}$ replaces the true mean term μ and n represents the number of samples.

$$SD = \sqrt{\frac{\sum (xi - \bar{x})^2}{n}}$$

Eq. 2.3

If the number of replicate determinations is small (about 30 or less), which is common with most assays, the n is replaced by the $n-1$ term, and **Equation [2.5]** is used. Unless you know otherwise, **Equation [2.4]** is always used in calculating the standard deviation of a group of assays.

$$SD = \sqrt{\frac{\sum (xi - \bar{x})^2}{n - 1}}$$

Eq. 2.4

Table 2.1: Determination of the Standard Deviation of Percent Moisture in processed cheese spread

Measurement	Observed moisture (%)	Deviation from the mean ($x_i - \bar{x}$)	$(x_i - \bar{x})^2$
1	45.22	+0.05	0.0025
2	45.45	+0.28	0.0784
3	45.10	-0.07	0.0049
4	44.91	-0.26	0.0676
$\Sigma x_i = 180.68$			$\Sigma (x_i - \bar{x})^2 = 0.153$

$$\bar{x} = \frac{\Sigma x_i}{n} = \frac{180.68}{4} = 45.17$$

$$SD = \sqrt{\frac{\Sigma (x_i - \bar{x})^2}{n - 1}} = \sqrt{\frac{0.153}{3}} = 0.0511$$

$$\text{Coefficient of variation (CV)} = \frac{SD}{\bar{x}} \times 100 \%$$

Eq. 2.5

$$CV = \frac{0.0511}{45.17} \times 100 \% = 0.113$$

The CV tells us that our standard deviation is only **0.113%** as large as the mean. For our example, that number is small, which indicates a high level of precision or reproducibility of the replicates. As a rule, a CV **below 5%** is considered **acceptable**, although it depends on the type of analysis.

SUMMARY

The results of an analytical study are only as good as the data used, but the data are only as good as the thoroughness displayed by the analyst in performing the measurements and minimizing errors. An analysis always takes less time to do once properly than to do over again because of carelessness or excessive speed.

Chapter 3

Sampling and Sample preparation

Objectives

- After reading this chapter, you should be able to:
 - Integrate Sampling and treatment of food samples for proximate analysis.
 - Explain sampling plan and sampling procedures.

Introduction

Quality attributes in food products, raw materials, or ingredients are measurable characteristics that need **monitoring** to ensure that specifications are met. In most cases, quality attributes are measured on small portions of material that are taken periodically from continuous processes or on a certain number of small portions taken from a lot. The small portions taken for analysis are referred to as **samples**, and the entire lot or the entire production for a certain period of time, in the case of continuous processes, is called a **population**. The process of taking samples from a population called **sampling**. If the procedure done correctly, the measurable characteristics obtained for the samples become a very accurate estimation of the population.

✚ If the sample is **not prepared properly** for analysis, or if the components become altered during preparation, the **results will be inaccurate** regardless of the effort, the precision of the apparatus, and the techniques used in the analysis. The aim of sample preparation is to mix a large sample in the laboratory. Moreover, a representative piece of material is extracted from a larger amount. However, sample size is limited by time, cost, sampling methods, and the logistics of sample handling, analysis, and data processing.

✚ There are three steps in sample preparation for chemical analysis of foods: sampling, homogenization, and sample preparation (**Figure 3.1**). The aim of each of the three steps is to increase the **accuracy** and **precision** of the analysis. At the same time, each step introduces inherent errors.

Sample preparation includes some steps. These steps depend on the sample, the matrix, and the concentration level at which the analysis needs to be carried out. After the sample preparation is complete, the analysis can be carried out.

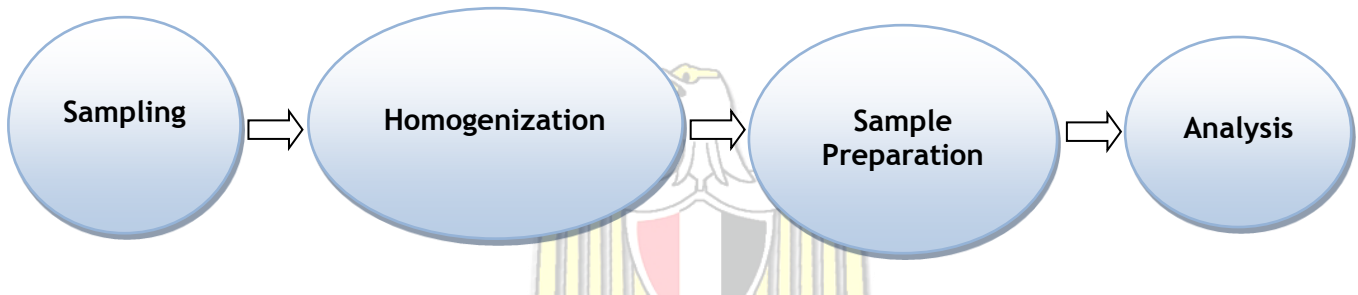


Fig. 3.1 Steps in a measurement process

Purpose of Analysis:

The first thing to decide when choosing a suitable sampling plan is the purpose of the analysis. Samples are analyzed for a number of different reasons in the food industry and this affects the type of **sampling plan used**:

- **Official samples.** Samples may be selected for official or legal requirements by government laboratories. These samples are analyzed to ensure that manufacturers are supplying safe foods that meet legal and labeling requirements. An officially sanctioned sampling plan and analytical protocol is often required for this type of analysis.
- **Raw materials.** Raw materials are often analyzed before acceptance by a factory, or before use in a particular manufacturing process, to ensure that they are of an appropriate quality.
- **Process control samples.** A food is often analyzed during processing to ensure that the process is operating in an efficient manner. Thus, if a problem develops during processing it can be quickly detected and the process adjusted so that the properties of the sample are not adversely affected. Techniques used to monitor process control must be capable of producing precise results in a short time. Manufacturers can either use analytical techniques that measure the properties of foods on-line, or they can select and remove samples and test them in a quality assurance laboratory.
- **Finished products.** Samples of the final product are usually selected and tested to ensure that the food is safe, meets legal and labeling requirements, and is of a high and consistent quality. Officially sanctioned methods are often used for determining nutritional labeling.
- **Research and Development (R&D).** Samples are analyzed by food scientists involved in fundamental research or in product development. In many situations it is not necessary

to use a sampling plan in R&D because only small amounts of materials with well-defined properties are analyzed.

Sampling Plan

Most sampling is done for a specific purpose, and the purpose may dictate the nature of the sampling approach. The two primary objectives of sampling are often to estimate the average value of a characteristic and determine if the average value meets the specifications defined in the sampling plan. Sampling purposes vary widely among different food industries; however, the **most important categories include the following:**

1. Nutritional labeling
2. Detection of contaminants and foreign matter
3. Statistical process control (Quality Assurance)
4. Acceptance of raw materials, ingredients, or products (Acceptance Sampling)
5. Release of lots of finished product
6. Detection of adulterations
7. Microbiological safety
8. Authenticity of food ingredients, etc.

The International Union of Pure and Applied Chemistry (IUPAC) defines a **sampling plan** as “A predetermined procedure for the selection, withdrawal, preservation, transportation, and preparation of the portions to be removed from a lot as samples”. Depending on the purpose of the sampling plan, samples are taken at different points of the food production system, and the sampling plan may vary significantly for each point.

▪ The choice of a sampling plan is an important consideration, especially when monitoring **food safety** by measurement of fungal toxins, named **mycotoxins**, in food systems. Mycotoxins are distributed broadly and randomly within a population and a normal distribution cannot be assumed. Such distribution requires a combination of many randomly selected portions to obtain a reasonable estimate of mycotoxin levels. Methods of analysis that are extremely precise are not needed when determining mycotoxin levels, when sampling error is many times greater than analytical error. In this case, sampling and good comminution and mixing prior to particle size reduction are more important than the chemical analysis itself.

Sampling Procedures

Details for the sampling of specific food products are described in the Official Methods of Analysis of AOAC International and in the Code of Federal Regulations (CFR).

The AOAC method 939.14 for eggs and egg products (AOAC 1990). Samples are taken from representative number of containers in lot. Sampling tube or dipper, auger, spoon, and hatcher are sterilized by wiping with alcohol-soaked cotton and flaming over alcohol lamp or another burner. Between sampling, instruments are washed, dried, and re-sterilized. Open and sample all containers under as nearly aseptic conditions as possible. For example, liquid eggs are mixed in the container with sterile sample tube or dipper, and transfer to sterile sample container. Keep samples at $<5^{\circ}\text{C}$ but avoid freezing. Odor of each container sampled is observed and recorded as normal, abnormal, reject, or musty.

The AOAC Method 925.08 (2007) describes the method for **sampling flour from sacks**. Sampling is done by drawing a core from a corner at the top of the sack diagonally to the center. The sampling instrument is a cylindrical, polished trier with a pointed end. It is 13mm in diameter with a slit at least one-third of the circumference of the trier. A second sample is taken from the opposite corner in a similar manner. The cores are stored for analysis in a clean, dry, airtight container that has been opened near the lot to be sampled. The container should be sealed immediately after the sample is added. A separate container is used for each sack.

Homogeneous vs. Heterogeneous Populations

The ideal population would be uniform throughout and identical at all locations. Such a population would be homogeneous. Sampling from such a population is simple, as a sample can be taken from any location and the analytical data obtained will be representative of the whole. However, this occurs rarely, as even in an apparently uniform product, such as sugar syrup, suspended particles and sediments in a few places may render the population heterogeneous. In fact, most populations that are sampled are heterogeneous. Therefore, the location within a population where a sample is taken will affect the subsequent data obtained. However, sampling plans and sample preparation can make the sample representative of the population or take heterogeneity into account in some other way.

Manual vs. Continuous Sampling

To obtain a **manual sample**, the person taking the sample must attempt to take a “random sample” to avoid human bias in the sampling method. Thus, the sample must be taken from a number of locations within the population to ensure that it is representative of the whole population. For liquids in small containers, this can be done by shaking prior to sampling. When

sampling from a large volume of liquid, such as that stored in silos, aeration ensures a homogeneous unit. Liquids may be sampled by pipetting, pumping, or dipping.

Continuous sampling is performed mechanically. **Figure 3.2** shows an automatic sampling device that is used to take liquid samples from a continuous production line. Continuous sampling should be less prone to human bias than manual sampling.



Fig. 3.2 An automatic liquid sampling device that uses air under high pressure to collect multiple 1.5-ml samples. The control box (*left*) regulates the sampling frequency. (Courtesy of Liquid Sampling Systems Inc., Cedar Rapids, IA.)

Preparation of Samples

1. Particle Size Reduction

If the particle size or mass of the sample is too large for analysis, it must be reduced in bulk or particle size. To obtain a smaller quantity for analysis the sample can be spread on a clean surface and divided into quarters. The two opposite quarters are combined. If the mass is still too large for analysis, the process is repeated until an appropriate amount is obtained. This method can be modified for homogeneous liquids by pouring into four containers, and it can be automated (**Figure 3.3**). The samples are thus homogenized to ensure negligible differences between each portion.

An example of size reduction is the preparation of solid sugar products for analysis as described in **AOAC Method 920.175 (2007)**. The method prescribes that the sugar should be ground, if necessary, and mixed to uniformity. Raw sugars should be mixed thoroughly and rapidly with a spatula. Lumps are to be broken by a mortar and pestle or by crushing with a glass or iron rolling pin on a glass plate.

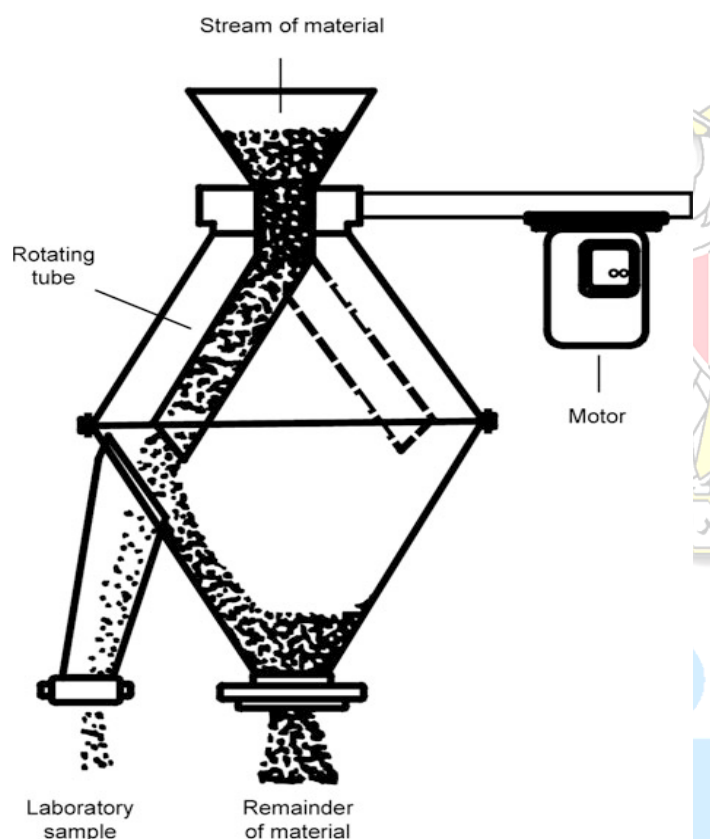


Fig. 3.3 A rotating tube divider for reducing a large sample (ca. 880 kg) of dry, free-flowing material to a laboratory size sample

2. Grinding

Grinding is important both for sample preparation prior to analysis and for food ingredient processing. Various mills are available for reducing particle size to achieve sample homogenization. To homogenize moist samples, bowl cutters, meat mincers, tissue grinders, mortars and pestles, or blenders are used, while mortars and pestles and mills are best for dry samples. Some foods are more easily ground after drying in a desiccator or vacuum oven. Grinding wet samples may cause significant losses of moisture and chemical changes. In contrast, grinding frozen samples reduces undesirable changes. The grinding process **should not** heat the sample, and therefore the grinder should not be overloaded because heat will be produced through friction. Contact of food with bare metal surfaces should be avoided if trace metal analysis is to be performed.

Grinding Equipment:



Fig. 3.4 Laboratory mills for sample preparation, Cyclone mill (A&B) and roller mill (C)
 Example of samples: Cereals, feed, legumes.
 -Rapid sample preparation of high-fat, high-moisture and fibrous samples (D) such as oilseeds, prepared foods, meat products, fruit, vegetables, grains, seeds and feed
 -Homogenizer for grinding and mixing food sample (E) as meat, fish, fruit, vegetables.

Preventing Changes in Sample

Once we have selected our sample we have to ensure that it does not undergo any significant changes in its properties from the moment of sampling to the time when the actual analysis is carried out, e.g., enzymatic, chemical, microbial or physical changes. There are a number of ways these changes can be prevented.

- **Enzymatic Inactivation:** Many foods contain active enzymes they can cause changes in the properties of the food prior to analysis, e.g., proteases, cellulases, lipases, etc. If the action of one of these enzymes alters the characteristics of the compound being analyzed, then it will lead to erroneous data and it should therefore be **inactivated** or **eliminated**. Freezing, drying, heat treatment and chemical preservatives (or a combination) are often used to control enzyme activity, with the method used depending on the type of food being analyzed and the purpose of the analysis.
- **Lipid Protection:** Unsaturated lipids may be altered by various oxidation reactions. Exposure to light, elevated temperatures, oxygen or pro-oxidants can **increase** the rate at which these reactions proceed. Consequently, it is usually necessary to store samples that have high unsaturated lipid contents under nitrogen or some other inert gas, in dark rooms or covered bottles and in refrigerated temperatures. Providing that they do not interfere with the analysis **antioxidants** may be added to retard oxidation.
- **Microbial Growth and Contamination:** Microorganisms are present naturally in many foods and if they are not controlled they can alter the composition of the sample to be analyzed. Freezing, drying, heat treatment and chemical preservatives (or a combination) are often used to control the growth of microbes in foods.
- **Physical Changes:** A number of physical changes may occur in a sample, e.g., water may be lost due to evaporation or gained due to condensation; fat or ice may melt or crystallize; structural properties may be disturbed. Physical changes can be **minimized** by controlling the temperature of the sample, and the forces that it experiences.

Identification

Laboratory samples should always be labeled carefully so that if any problem develops its origin can easily be identified. The information used to **identify a sample** includes:

- a) Sample description
- b) Time of take sample
- c) Location of sample
- d) Person who took the sample.
- e) Method used to select the sample.

Sample Storage

Effect of sample storage and preparation on nutrient content and precautions required to minimize that effect as shown in **table 3.1**

Table 3.1 Effects of sample storage and preparation on nutrient content

Effects	Potential Changes	Nutrients Affected	Precaution
Drying out	Loss of water	All nutrients	Design of protocol Keep samples sealed, weigh food at start and during preservation
Absorption	Gain of water	All nutrients	Design of protocol keep samples in sealed container
Oxidation	Destruction of unsaturated fatty acids, loss of vitamins	Alterations in profile of fats	Store at -30°C in sealed containers under nitrogen. Add antioxidants, bacteriostatic agents
Light	Photo degradation	Loss of riboflavin	Protect from light
Contamination during sampling	From cooking vessels, soil, dust	Increase inorganic Nutrients	Design protocol to minimize contamination, gently rinse with distilled water
Contamination from metallic blades, glassware	Increase in inorganic nutrients	Increase in major trace elements	Select apparatus with care Clean all utensils Store in plastic bags
Separation	Separation of fats	Changes in Compositional. Alteration in fiber content	Avoid over vigorous mixing and thaw/freeze cycles

Sources of errors in sampling

During handling of samples may be occur some errors which are listed in **table 3.2**

Table 3.2 Major sources of errors in sampling

Source	Examples	Precaution
Food sample identification	Poor labeling of samples	Maintenance of documentation throughout sampling and analytical process
Nature of sample	Samples do not conform to the defined sampling protocol	Explicit instructions, sampling protocol, training of sample staff
Transport and handling	Samples contaminated, degraded or depleted during transport, loss of samples	Protocol specifies condition to be maintained, supervision
Analytical sample preparation	Incorrect mixing or homogenization	Proper supervision in laboratory Laboratory quality assurance
Analytical sample storage	Incorrect storage of samples	Proper laboratory techniques and supervision

Things to note

- If products are in bulk: storage procedures, choice of containers, modes of transport should be considered.
- Use containers that are clean, dry, leak-proof, wide-mouthed, sterile, and of a size suitable for samples of the product.
- Whenever possible, avoid glass containers, which may break.
- For dry materials, use sterile metal boxes, cans, bags, or packets with suitable closures.
- Transport frozen or refrigerated products in approved insulated containers of rigid construction.
- **Homogeneous foods:**
 - ▣ **Solids:**
 - ▣ Friable: crumble and mix.
 - ▣ Sticky: freeze and crush at low temperature.
 - ▣ Hygroscopic: take portions rapidly into pre-weighed sealable containers for weighing.
 - ▣ **Emulsions:**
 - ▣ Take by weight rather than volume; warm and mix.
 - ▣ **Liquids with suspended solids:**
 - ▣ Homogenize, or sample during gentle mixing.
- The analyst should always keep a detailed notebook clearly documenting the sample selection and preparation procedures performed and recording the results of any analytical procedures carried out on each sample.
- Each sample should be marked with a code on its label that can be correlated to the notebook. Thus, if any problem arises, it can easily be identified.

Chapter 4

Physicochemical Properties of Foods

Objectives

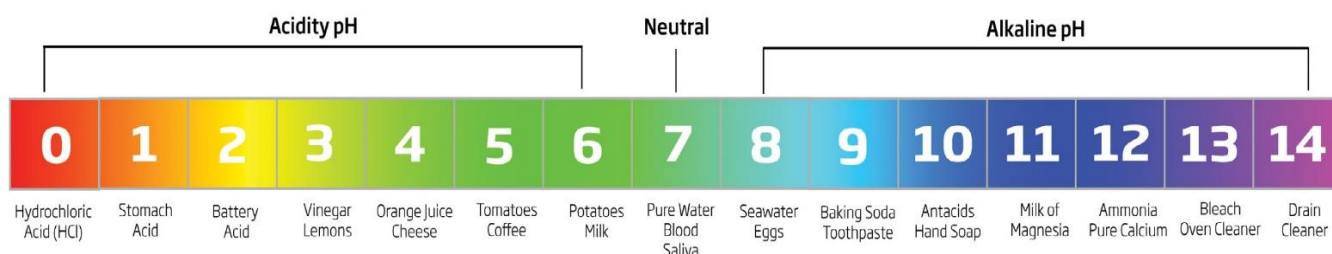
- After reading this chapter, you should be able to:
 - Explain pH of food as well as the steps of measurement and calibration of the device.
 - Identify the physical properties, including rheological (viscometer) and mechanical properties (texture analyzer) of food.

Guide to Measuring pH of Foods

What is pH?

In technical terms, pH is the hydrogen ion activity in a solution. The equation is commonly written as $\text{pH} = -\log [\text{H}^+]$.

This equation will determine pH according to a scale from pH 0 to 14, with solutions less than pH 7 being acidic and solutions greater than 7 being basic. A pH of 7 is neutral and is neither an acid nor a base.



How does pH affect food quality?

Flavor	Organic acids, such as citric acid, can provide a tart or sour flavor to foods.
Texture	Texture is particularly susceptible to pH changes. Low pH will result in a cheese without shape or hold while high pH causes cheese to crumble.
Fermentation	pH affects bacteria used in food production to make cheese, yoghurt, vinegar, and soy sauce, to name just a few. Yeast performs best at a pH of 4.5-6.0.
Appearance	pH plays a role in haze formation and contributes to pigment. Anthocyanins, the red pigments found in berries, turn blue, green, or yellow in alkaline conditions.
Shelf stability	pH prevents spoilage by inhibiting bacterial growth. Bacteria, such as <i>E. coli</i> , require pH higher than 4.6 to thrive; anything lower will inhibit growth.

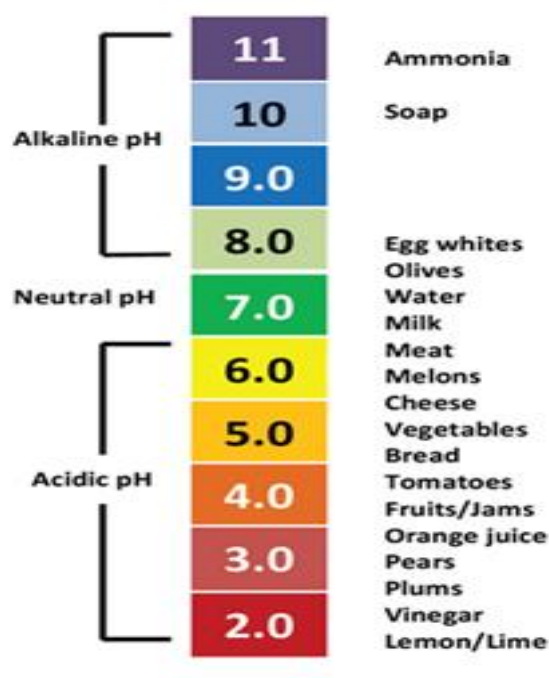


Fig. 4.1 Classification of foods based on pH



Fig. 4.2 pH meter

Methodology for pH measurements:

- Use commercially prepared buffer.
- pH meter with accuracy to be approximately + 0.1 pH unit.
- Determine pH of samples at room temperature (25 ° C).

What you need?

1. pH meter
2. pH electrode
3. Calibration Solutions

How to calibrate and use a pH meter?

1- Preparing for calibration:

- Turn on your pH meter.** Before you begin to calibrate and use your pH meter you will first need to turn it on and allow adequate time for the meter to warm up. This should generally take around 30 minutes but check your pH meter's operating manual for exact times.
- Clean your electrode.** Take the electrode out of its storage solution and rinse it with distilled water under an empty waste beaker. Once rinsed, blot dry with Kim-wipes. **Prepare your buffers.** You will generally need more than one buffer for calibrating a pH meter. The first will be a "neutral" buffer with a pH of 7, and the second should be near the expected sample pH, either a pH of 4 or 9.21. Buffers with a higher pH (9.21) are best for measuring bases, whereas buffers with a low pH (4) are best for measuring acidic samples. Once you have chosen your buffers allow them to reach the same temperature as the pH meter because pH readings are temperature dependent.
 - Pour your buffers into individual beakers for calibration.

- Buffers should be kept in a beaker for no longer than two hours.
- Discard the buffer when you are finished. Do not return it to its original container

2- Calibrating pH Meter (Practical)

- A. **Place your electrode in the buffer with a pH value of 7 and begin reading.** Press the “measure” or calibrate button to begin reading the pH once your electrode is placed in the buffer.
 - Allow the pH reading to stabilize before letting it sit for approximately 1-2 minutes.
- B. **Set the pH.** Once you have a stable reading, set the pH meter to the value of the buffer’s pH by pressing the measure button a second time. Setting the pH meter once the reading has stabilized will allow for more accurate and tuned readings.
- C. **Rinse your electrode with distilled water.** Rinse and pat dry with a lint-free tissue, like Kim-wipes, in between buffers.
- D. **Place your electrode in the appropriate buffer for your sample and begin reading.** Press the measure button to begin reading the pH once your electrode is placed in the buffer.
- E. **Set the pH a second time.** Once your reading has stabilized, set the pH meter to the value of the buffer’s pH by pressing the measure button.
- F. **Rinse your electrode.** You can use distilled water to rinse. Use a lint-free tissue, as Kim-wipes, in between buffers to dry the electrode.



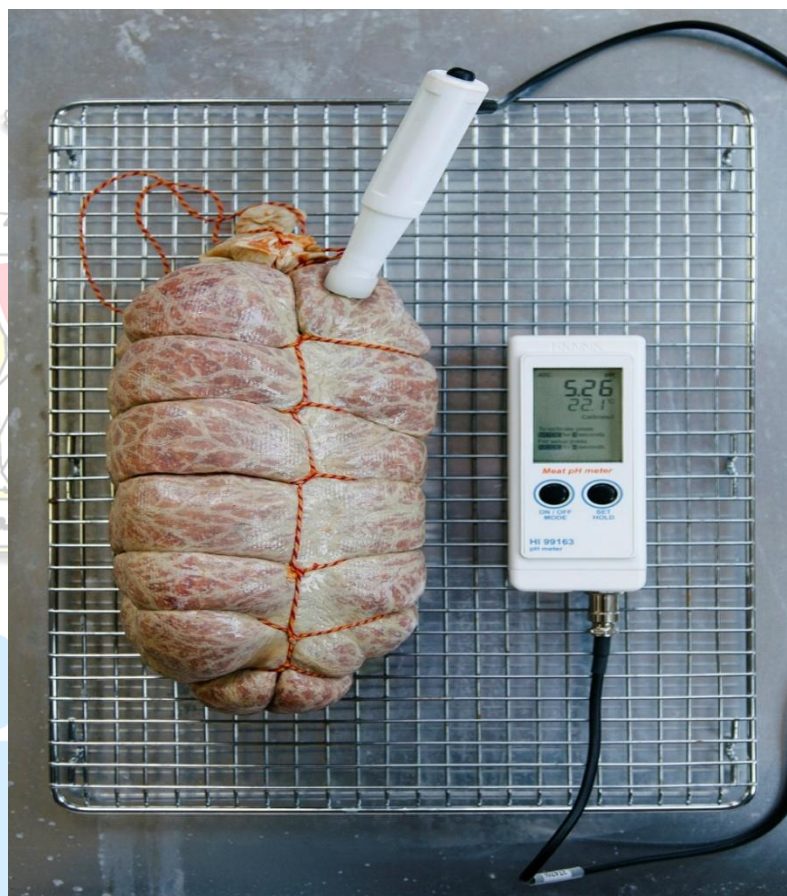
Fig. 4.3 Calibration Process of pH meter

Direct vs. Sample Testing

There are **two primary methods** for testing the pH in food:

Direct Testing

Some foods can be tested by taking a pH reading directly from the food product. For example, testing a salami, cheese, meat, and yoghurt. This is usually done with a conical-tip electrode with a plastic (PVDF) body. The electrode is inserted directly into the product and a measurement is taken. These electrodes almost always have food-grade electrolytes or double junctions, so the exchange of electrolyte or silver into the product is not a concern. This type of testing only applies to samples that have uniform consistency and/or are not composed of liquid and solid parts.



Sample Testing

The second method of testing is by taking a portion or sample of the food, testing, and then discarding the sample.

Slurry Method

A form of sample testing, the Slurry Method, involves the dilution of a food sample with deionized/distilled water, referred to as creating a “slurry”. As pH is the testing of hydrogen ion activity, distilled or deionized water does not greatly affect pH readings, as there is not a significant amount of ions present.

Benefits

A slurry can be beneficial when you are testing a semi-solid or solid with a spherical-tip electrode, as it allows the sample to surround the electrode and read more accurately.

FDA Dilution Amount

The FDA suggests that a sample for pH testing not be diluted with more than 16.67% deionized/distilled water; that is, a 50g sample into 10g of water, representing a **5:1 sample-to-deionized water ratio**.

Recommended Slurry Ratio

A 1:2 ratio of sample-to-deionized water (e.g. a 50g sample to 100g deionized water) should, in most cases, provide sufficient dilution to achieve a sample solution capable of testing with a spherical-bulb electrode.

How to create a slurry

1. Prepare 50g of your semi-solid or solid sample
2. Prepare 100g of deionized water
3. Puree the sample with the deionized water until a uniform paste is achieved. If there is not sufficient liquid to create a thin paste, the sample may be diluted with up to 10x its weight with deionized water without affecting the pH level (e.g. 50g sample diluted with 500g water). Use as little water as necessary to achieve desired consistency.
4. Test using the corresponding method **that follows**

pH Testing Methods (Practical)

Liquids

Example Foods: Juice, milk, vinegar, etc.

Testing Method

1. Ensure that the electrode is calibrated.
2. Either pull a sample or test directly in the food product/batch.
3. Rinse the electrode with deionized or distilled water.
4. Submerge the electrode in the sample, stirring to ensure a consistent sampling (a magnetic stirrer is ideal); leave until a stable reading is reached, and record pH.
5. Rinse the electrode.
6. Take another reading and make certain that the second reading agrees with the first reading, as this ensures that the mixture is homogeneous.
7. Rinse and clean electrode.

Solid Foods

Example Foods: Raw meats, salami, cheese, etc.

Testing Method

1. Ensure that the electrode is calibrated.
2. Either pull a sample or test directly in the product.
3. Rinse the electrode with deionized or distilled water.
4. Insert the electrode in the sample; leave until a stable reading is reached, and record pH.
5. Rinse the electrode.
6. Take another reading and make certain that the second reading agrees with the first reading.

7. Rinse and clean electrode.

Semisolid Foods

If consistency is uniform

Example Foods: Pudding, bread dough, etc.

Testing Method

1. Ensure that the electrode is calibrated.
2. Either pull a sample or test directly in the product/batch.
3. Rinse the electrode with deionized or distilled water.
4. Submerge the electrode in the sample, leave until a stable reading is reached, and record pH
5. Rinse the electrode.
6. Take another reading and make certain the second reading agrees with the first reading; this ensures that the mixture is homogeneous.
7. Rinse and clean electrode.

If consistency is not uniform

Example Foods: Potato salad, egg salad, chunky tomatoes in sauce, etc.

Testing Method

1. Ensure that the electrode is calibrated.
2. Pull a sample; do not test directly in batch.
3. Puree the sample to a uniform consistency; if there is not sufficient liquid to create a thin paste, the sample may be diluted with up to 10x its weight with deionized water without affecting the pH level (e.g. 50g sample diluted with 500g water). Use as little water as necessary to achieve desired consistency.
4. Rinse the electrode with deionized or distilled water.
5. Submerge the electrode in the sample; leave until a stable reading is reached, and record pH
6. Rinse the electrode.
7. Take another reading to make certain that the second reading agrees with the first reading. This ensures that the mixture is homogeneous.
8. Rinse and clean electrode.

Oil-Marinated Foods

Example Foods: Olives in olive oil, fish in oil, etc.

Testing Method

1. Ensure that the electrode is calibrated.
2. Pull a sample.
3. Strain and separate the solids from the oil. Oil may be discarded, as it does not affect pH.
4. Puree the sample to a uniform consistency; if there is not sufficient liquid to create a thin paste, the sample may be diluted with up to 10x its weight with deionized water without affecting the pH level (e.g. 50g sample diluted with 500g water). Use as little water as necessary to achieve desired consistency.
5. Rinse the electrode with deionized or distilled water.
6. Submerge the electrode in the sample; leave until a stable reading is reached, and record pH
7. Rinse the electrode.
8. Take another reading to make certain that the second reading agrees with the first reading. This ensures that the mixture is homogeneous.
9. Rinse and clean electrode.

Rheological and Textural Properties of Foods

Rheology, by name, originated from a combination of two Greek words “**rheo**” meaning flow and “**logy**” meaning science. Therefore, rheology can be defined as the science of deformation and flow of matters. Food materials can be categorized in three main groups: **fluid, visco-elastic, and solid materials**. They exhibit flow and deformation under external forces. If the material is an elastic solid, the applied force will not result in a flow but in a deformation; if it is a viscous material it will start to flow with the application of a force.

Whether working in product development, quality control, or process design and scale-up, rheology plays an integral role in the manufacture of the best products. Determination and control of the flow properties of fluid foods is **critical** for optimizing processing conditions and obtaining the desired beneficial effects for the consumer. Transportation of fluids (pumping) from one location to another requires pumps, piping, and fittings such as valves, elbows, and tees. Proper sizing of this equipment depends on a number of elements but primarily on the flow properties of the product. For example, the equipment used to pump a dough mixture would be very different from that used for milk. Additionally, rheological properties are fundamental to many aspects of food safety. During continuous thermal processing of fluid foods, the amount of time the food is in the system (known as the **residence time or RT**), and therefore the amount of heating or “thermal dose” received, relates directly to its flow properties.

Viscosity of Foods

Viscosity is an important property of fluid foods. It is defined as the **internal friction of a liquid or its ability to resist flow**. As viscosity changes the flow properties of a liquid food and influences the appearance and the consistency of a product, this measuring variable is important in most production stages. Viscosity is also a characteristic of the **texture** of food. This means that the viscosity of a product **must be controlled** and measured in production so that each batch is consistent from day to day.

Definitions:

Laminar Flow and Turbulent Flow:

Laminar flow is streamline flow in a fluid. **Turbulent flow** is fluid flow in which the velocity varies erratically in magnitude and direction.

The difference between laminar flow and turbulent flow is illustrated in Fig. 4.4. Suppose a fluid is being pumped through a pipe at a constant rate and a thin thread of colored solution is injected into the flowing stream. If laminar flow is occurring, the thread of colored solution will move straight down the tube. In the case of turbulent flow there are many eddies and currents, which are shown by the line of colored solution breaking up and forming eddies and vortices as it moves down the pipe. Laminar flow occurs at **slow rates of flow** and turbulent flow occurs at **high rates of flow**.

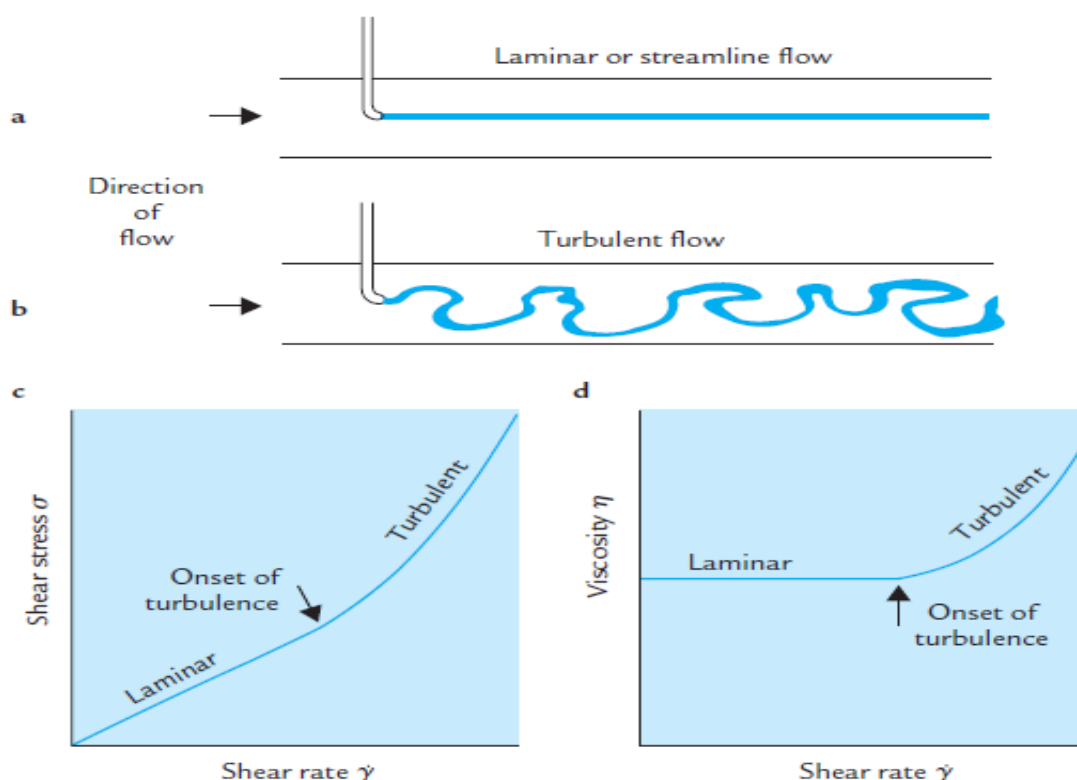


Fig.4.4. The difference between (a) laminar and (b) turbulent flow; (c) shear stress versus shear rate for a Newtonian fluid in the laminar and turbulent flow range; (d) viscosity versus shear rate for a Newtonian fluid in the laminar and turbulent flow range.

Dynamic Viscosity

Dynamic viscosity, which is frequently, called '**viscosity**,' or '**absolute viscosity**,' is the internal friction of a liquid or its tendency to resist flow. It is usually denoted by η and is defined by the equation 4.1:

$$\eta = \sigma / \dot{\gamma} \quad \text{Eq. 4.1}$$

Where η is the viscosity; σ , the shear stress; and, $\dot{\gamma}$, the shear rate.

According to the International Organization for Standardization (ISO) the unit of measurement for dynamic viscosity is the **pascal second (Pa.s)**. Since the pascal second is a large unit of measurement, a more common unit for low viscosity fluids is the **millipascal second (mPa.s)** where $1000 \text{ mPa.s} = 1 \text{ Pa.s}$.

Kinematic Viscosity

This is defined as the absolute viscosity divided by the density of the fluid. It is usually denoted by ν :

$$\nu = \eta / \rho = \sigma / \rho \dot{\gamma} \quad \text{Eq. 4.2}$$

Where ν is the kinematic viscosity, η is the absolute viscosity and ρ is the density in grams per cubic centimeter. The unit for kinematic viscosity is the meter-square-second.

Relative Viscosity

This is sometimes called the '**viscosity ratio**,' which is the ratio of the viscosity of a solution to the viscosity of the pure solvent and is defined by the equation

$$\eta_{\text{rel}} = \eta / \eta_s \quad \text{Eq. 4.3}$$

Where η_{rel} is the relative viscosity; η , the viscosity of solution; and η_s , the viscosity of solvent.

Apparent Viscosity

This is the viscosity of a non-Newtonian fluid expressed as though it were a Newtonian fluid. It is a coefficient calculated from empirical data as if the fluid obeyed **Newton's law**.

Shear Stress

Shear stress is the stress component applied tangential to the plane on which the force acts. It is expressed in units of force per unit area. It is a force vector that possesses both magnitude and direction. The unit for shear stress is the pascal (Pa) with units of newton meter⁻² (Nm⁻²).

Shear Rate

Shear rate is the velocity gradient established in a fluid as a result of an applied shear stress. It is expressed in units of reciprocal seconds (s⁻¹).

Tests for viscous materials

Viscous materials can be deformed by shearing. Viscous behavior of a food material shows its ability to resist flowing. If a fluid flows between two parallel plates by applying shearing force (F) per unit area, the relative motion of the upper plate to lower plate causes the material to move with a velocity v (Figure 4.5).

If the material is a **Newtonian fluid** then the shearing stress (F/A) is directly proportional to the mean rate of shear

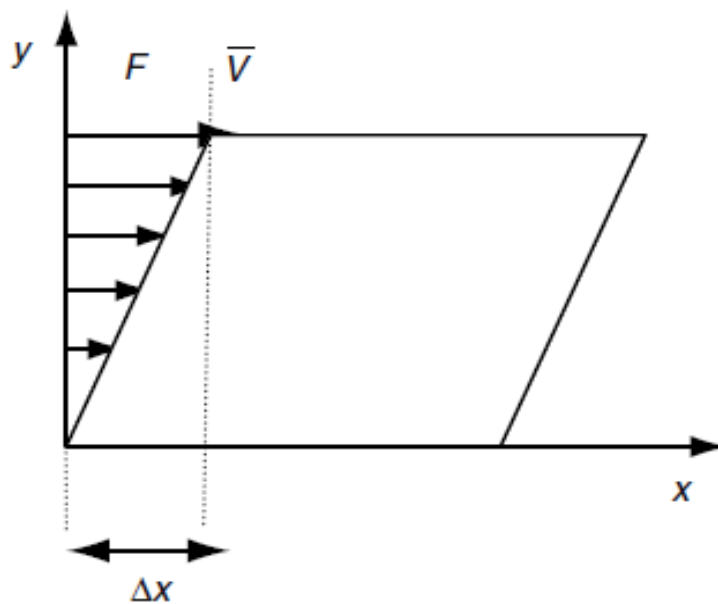


Fig.4.5 Simple shear flow

The General Equation for Viscosity

$$\eta = \frac{\sigma}{\dot{\gamma}}$$

Eq.4.4

Or

$$\sigma = \eta \dot{\gamma}$$

Eq. 4.5

Where

η refers to Newtonian viscosity or dynamic viscosity.

$\dot{\gamma}$ is the shear rate.

σ is the shear stress.

For **non-Newtonian fluids**, the relationship between shear stress and shear rate is **nonlinear** and the viscosity is a function of shear rate. The shear rate dependent viscosity is called **apparent viscosity** (η_a).

Figure 4.6 shows various types of fluids that exhibit this type of behavior. Time independent non-Newtonian fluids can be summarized as Bingham (plastic), pseudoplastic (shear thinning), dilatant fluid (shear thickening), pseudoplastic and dilatant fluids with a yield stress. Food materials like emulsions, gums, proteins, carbohydrates, and chocolate syrups are either pseudoplastic (shear thinning) or dilatant (shear thickening). Bingham fluids require an initial stress which is called “**yield stress**” to initiate the flow, and the shear stress versus shear rate plot is linear to it.

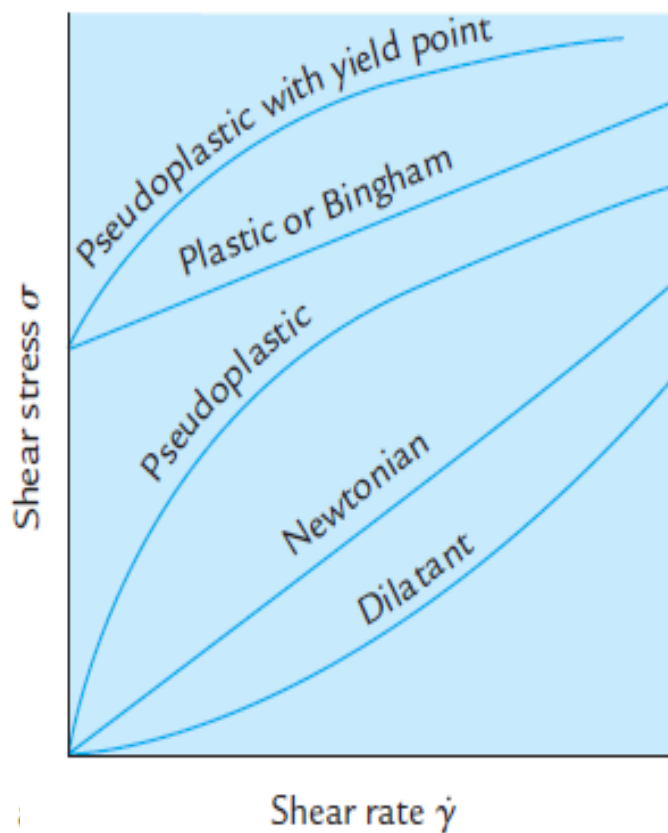


Fig.4.6 Time-independent flow behavior

This type of behavior is quite common in foods like mayonnaise, ketchup, and margarine. For pseudoplastic and dilatant fluids with a yield stress, these types of fluid behavior still require a yield stress, but above the yield stress the relationship between shear stress and shear rate is nonlinear.

Time-dependent non-Newtonian fluids can be grouped in four: thixotropic, rheopectic, shear thinning, and shear thickening (Figure 4.7). In **thixotropic fluids** (some starch-paste gels), the apparent viscosity decreases with time of shearing that is reversible. In **rheopectic fluids**, the apparent viscosity increases with time of shearing where the change is reversible. However, it is difficult to find food materials that exhibit this type of behavior. Some gum solutions and starch pastes are **shear-thinning fluids** where the apparent viscosity decreases with time with an irreversible change. The opposite of this behavior is **shear thickening** that can be seen in egg white or heavy cream where during shearing, viscosity increases irreversibly with time.

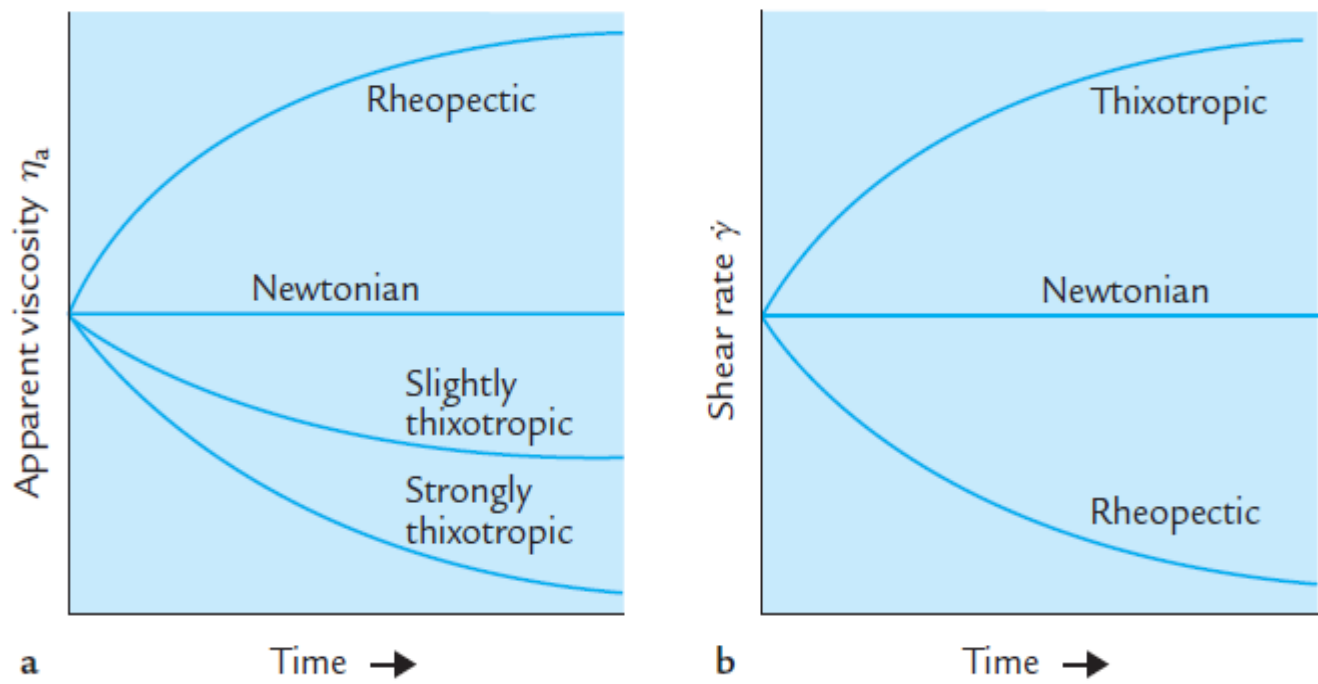


Fig. 4.7 Time dependency factors in fluid flow: (a) at constant rate of shear and (b) at constant shear stress.

Measurement Techniques for Fluid Behavior

Generally, rheometer, mixer, or relevant instruments like capillary viscometer and pipe setups can be used to analyze fluid behavior.

A capillary viscometer consists of a very small diameter and a cylindrical capillary tube where liquid is forced through the capillary by imposing a pressure drop. The ratio of the very small diameter of the tube and the very large length to diameter minimizes entrance and exit effects and ensures a fully developed velocity profile.

Some modification of a capillary rheometer where used long-entrance region and determined the pressure drops as the difference of two pressure values measured in the fully developed laminar region (Figure 4.8).

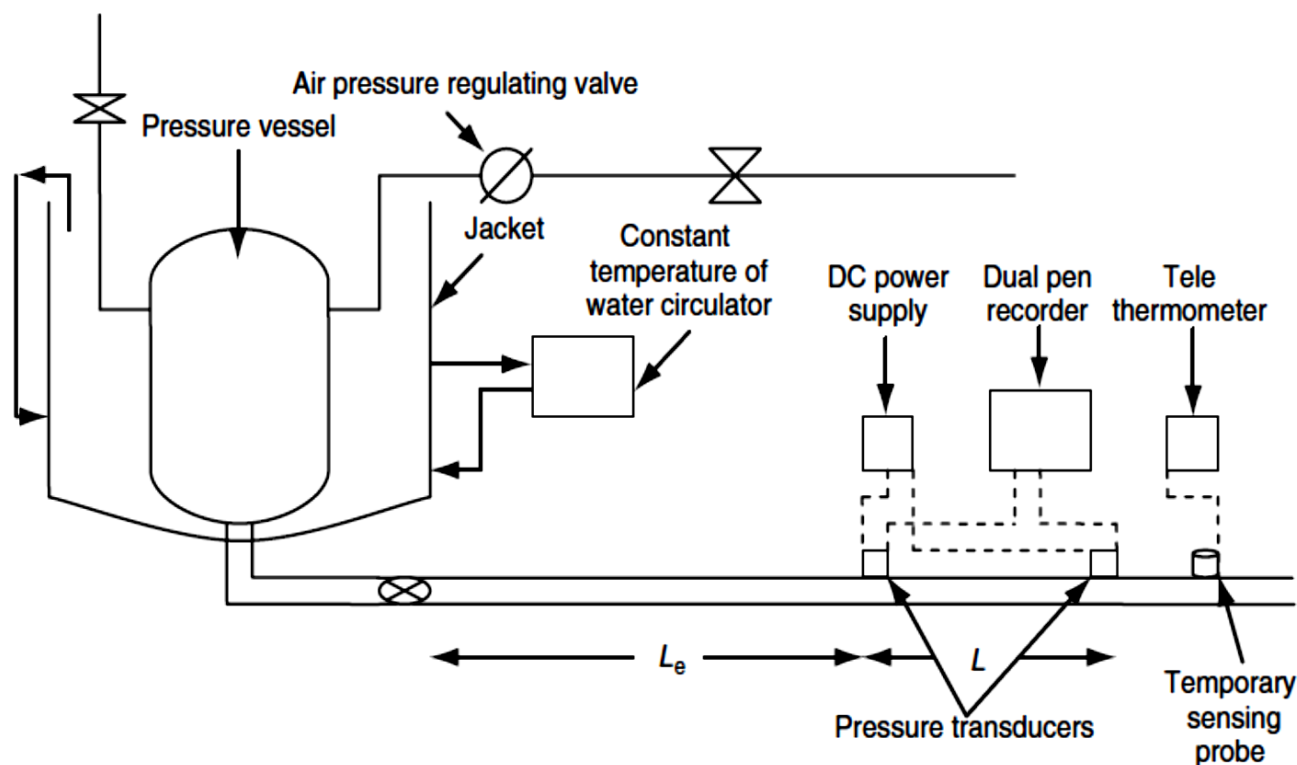


Fig. 4.8 Schematic diagram of the capillary setup.

Viscosity measurement methods for liquid food

A. Capillary Flow Viscometers

Capillary flow viscometers are generally in the form of a **U tube**. These types of viscometers are very simple, inexpensive and suitable for **low-viscosity fluids**. There are different designs of capillary viscometers. A typical design of capillary viscometer is shown in **Figure 4.9**.

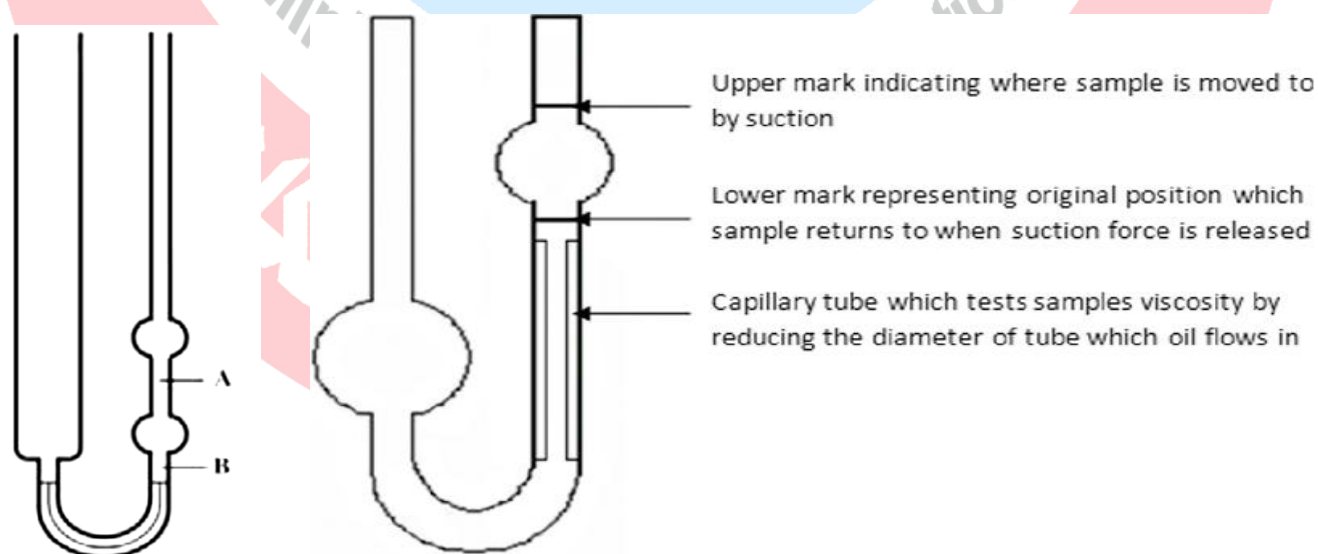


Fig.4.9 Cannon-Fenske capillary flow viscometer

The time for a standard volume of fluid to pass (**flow**) through a length of capillary tubing is measured. The driving pressure (causing the flow) is usually generated by the force of gravity acting on a column of the liquid, although it can be generated by the application of compressed air or by mechanical means. Glass capillary viscometers are widely used for **measuring low to medium viscosity Newtonian fluids** because of their high degree of accuracy, ease of operation and low cost. The diameter of a capillary viscometer should be small enough to provide **laminar flow**. Capillary viscometers are calibrated with Newtonian oils of known viscosities since the flow rate depends on the capillary radius, which is difficult to measure.

For the viscosity measurement, the viscometer is accurately filled with an accurately known volume of the test fluid and the apparatus is immersed in a constant temperature bath until equilibrium is reached. Then, fluid is sucked up from the other limb through the capillary tube until it is above the marked level (A) (**Fig. 4.9**). Then, suction is removed and fluid flows through the capillary tube under the influence of gravity or the induced pressure head and the time for the fluid to flow from mark **A to B** is recorded. This **time** is a direct measure of the **kinematic viscosity** since it depends on both viscosity and density of the fluid.

B. Tube Viscometers

This might be considered as a wide-bore capillary viscometer with a special capability to handle suspensions. It consists of a horizontal tube of uniform cross-sectional area that is usually 0.5 m long and may be even longer. The internal diameter typically ranges from 6 to 30 mm. Two pressure transducers are attached to the pipe to record the pressure drop over a given length of pipe. A constant pressure is maintained at the entrance of the tube that ensures laminar flow to occur and the volumetric flow rate is measured (**Figure 4.10**).

This type of viscometer was used to study the viscosity of pear, apricot and peach purees, apple sauce, grape juice, apple juice and their concentrates. It was also used to study the flow behavior of banana, guava, mango and papaya purees.

Tube viscometry method is currently used in order to determine rheological behavior of a product after a thermal treatment, but this technique requires a **large amount of material** and does not allow a **continuous measurement** of rheological properties during phase changes.

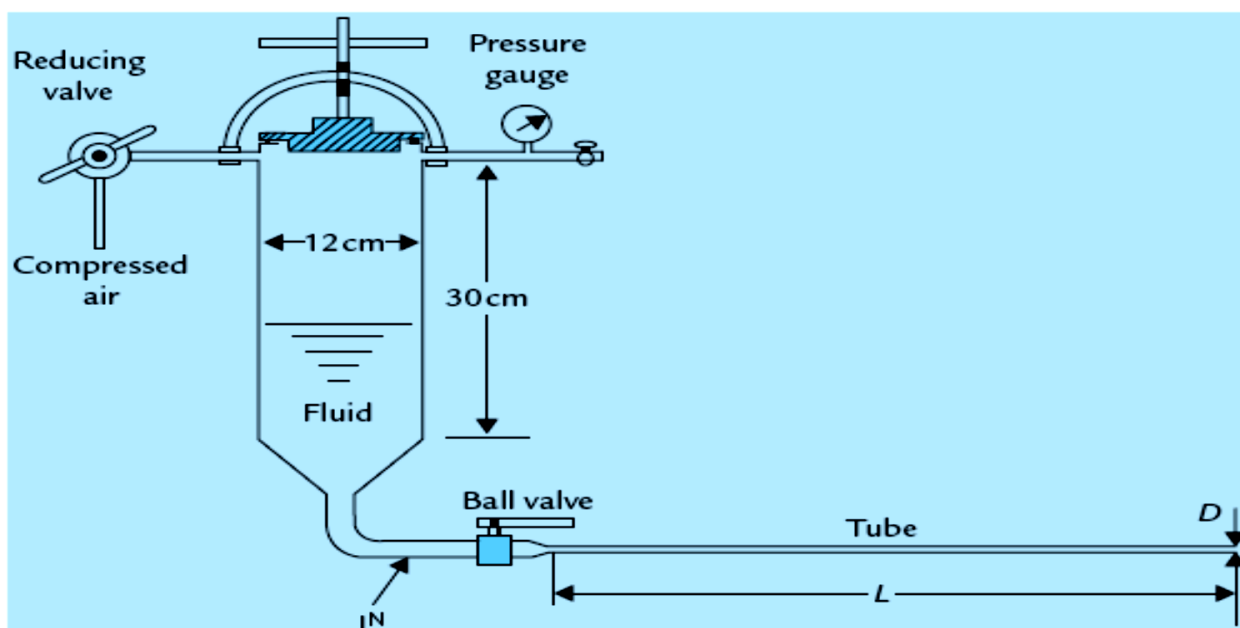


Fig. 4.10 Tube viscometer

C. Coaxial Rotational Viscometers (Concentric cylinder)

Coaxial rotational viscometers are also known as concentric cylinder or Couette viscometers. The principle is shown schematically in **Figure 4.11**. A bob that is circular in cross section is placed concentrically inside a cup containing the test fluid. Either the cup or the bob is rotated and the drag of the fluid on the bob is measured by means of some kind of torque sensor. The shear rate-shear stress relationship is the same whether the bob is rotated, and the cup held stationary or vice versa. This type of viscometer permits continuous measurements to be made under a given set of conditions and allows time-dependent effects to be studied. By changing the rate of shear or magnitude of stress it is possible to obtain viscosity measurements over a range of shearing conditions on the same sample. It can be used for **both Newtonian and non-Newtonian foods**. This is the most common type of viscometer that is used in the **food industry**. It might be called the 'workhorse' viscometer.

A rotational viscometer with two coaxial cylinders was used to investigate the rheological properties of food stabilizers. The rheological properties (shear stress and shear rate) of the puree at various temperatures were also studied with a rotational viscometer equipped with a coaxial cylinder measuring systems.

The major error that occurs in coaxial viscometers is the 'end effect' which arises from the drag of the fluid on the ends of the bob. The end effect of the top of the bob is easily eliminated by filling the cup to a level below that necessary to cover the bob. Since the top of the bob is not in contact with the liquid there is no drag. The end effect of the bottom of the bob can be determined experimentally by measuring the torque/angular-speed ratio with the cup filled to several different heights.

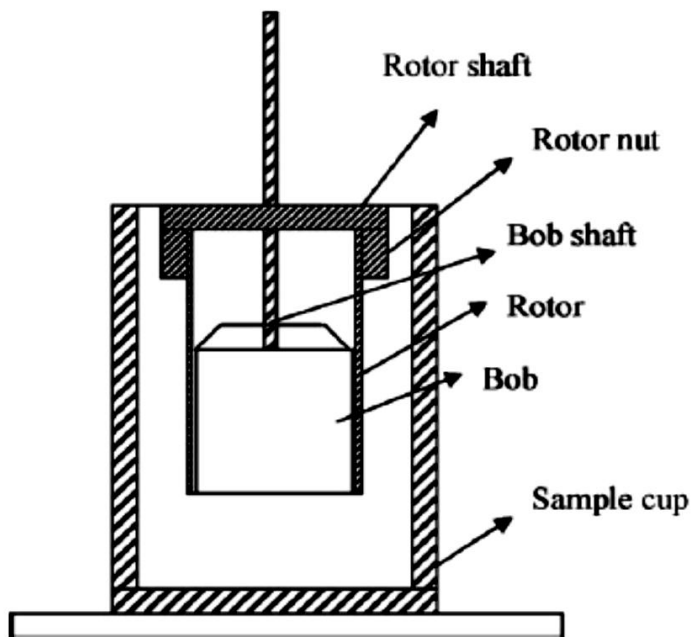


Fig. 4.11 Coaxial rotating cylinder viscometer

One of the most common rheological devices found in the food industry is the **Brookfield viscometer** (Figure 4.12). This affordable apparatus uses a spring as a torque sensor. The operator selects a rotational speed (rpm) of the bob, attached to the spring.



Fig. 4.12 Brookfield viscometer

D. Cone and plate viscometers

The operating principle for cone and plate viscometers is similar to that for concentric cylinder viscometers. The fluid is held by its own surface tension between a cone of small angle that just touches a flat surface (**Figure 4.13**). The torque caused by the drag of the fluid on the cone is measured as one of the members is rotated while the other member remains stationary. The special feature of the cone and plate viscometer is that the shear rate is uniform at all points in the fluid, provided that the angle of the cone is small. This makes the cone and plate viscometer of particular use for **non-Newtonian fluids**, because the true rate of shear can be obtained comparatively easily. Other features of this type of viscometer are (1) end effects are negligible, (2) a small amount of fluid is needed (usually less than 2 ml) and (3) the thin layer of fluid in contact with temperature-controlled metal base plate enables measurements to be made at high rates of shear without the need to compensate for the heating effect of the high shear rate.

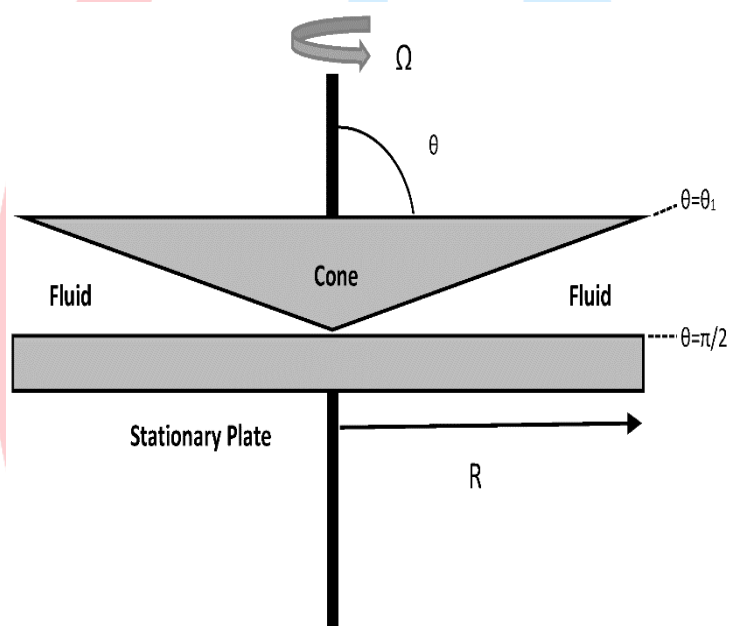


Fig. 4.13 Cone and plate viscometer

Table 4.1. Advantages and Disadvantages of Rotational Viscometry Attachments

Rotational Geometry	Advantages	Disadvantages
Concentric cylinder	Good for low-viscosity fluids Good for suspensions Large surface area increases sensitivity at low shear rates	Potential end effects Large sample required
Cone and plate	Constant shear stress and shear rate in gap Good for high shear rates Good for medium- and high-viscosity samples Small sample required Quick-and-easy cleanup	Large particles interfere with sensitivity Potential edge effects Must maintain constant gap height

E. Falling-Ball Viscometers

This type of viscometer operates on the principle of **measuring the time** for a ball to fall through a liquid under the influence of gravity. The falling ball reaches a limiting velocity when the acceleration due to the force of gravity is exactly compensated for by the friction of the fluid on the ball. This is a simple type of instrument that is useful for **Newtonian fluids** but has limited applicability to non-Newtonian fluids (**Figure 4.14**). It cannot be used for opaque fluids because the ball cannot be seen. This method is applicable when the diameter of the ball is so much smaller than the diameter of the tube through which it is falling that there is no influence of the wall on the rate of fall of the ball. A falling ball viscometer can be easily improvised in the laboratory. A large graduated glass cylinder is filled with the test fluid and a steel ball is gently dropped in the center of the cylinder. Sufficient distance of fall is allowed for the ball to reach the limiting velocity, and the fall of the ball is timed with a stopwatch. Steel ball bearings with a range of precisely controlled diameters can be obtained from engineering supply houses. The larger the ball, the faster it falls. Therefore, it is necessary to select a diameter ball that is small enough to fall at a rate that can be measured with some degree of accuracy with a stopwatch. The lower the density of the ball, the slower it falls. It is possible to obtain balls of material other than steel that have a different density. For example, glass marbles have a density of about 2.6 compared with 7.8 for steel. A glass marble will fall more slowly than a steel ball of equal size.



Fig.4.14 Falling- ball viscometer

Food Texture

The importance of texture in the overall acceptability of foods varies widely, depending upon the type of food. We could arbitrarily break it into three groups:

1. **Critical:** Foods in which texture is the dominant quality characteristic; for example, meat, potato chips, cornflakes and celery.
2. **Important:** Foods in which texture makes a significant but not a dominant contribution to the overall quality, contributing, more or less equally, with flavor and appearance; for example, most fruits, vegetables, cheeses, bread, most other cereal-based foods and candy fall into this category.
3. **Minor:** Foods in which texture makes a negligible contribution to the overall quality; examples are most beverages and thin soups.

There is an enormous range in textural characteristics of foods: the chewiness of bread crust and of meat, the softness of marshmallows, the crispness of celery and potato chips, the juiciness of fresh fruits, the smoothness and melting sensations of ice cream, the soft toughness of bread, the flakiness of fish, the crumbliness of cake, the melting of jelly, the viscosity of thick soup, the fluidity of milk, the thick smoothness of yogurt, the creaminess of pie topping and many others. This great range of types of rheological and textural properties found in foods arises from the human demand for variety in the nature of their food.

Table 4.2 Changes in Food Texture During Storage

Food	Texture change	Cause
Bread, crumb	Firmness increases, springiness decreases	Starch retrogradation, moisture transfer from starch to gluten.
Bread, crust	Crispness decreases, toughness increases	Moisture migrates from crumb to crust.
Butter and margarine	Firmness and graininess increase, spreadability decreases	Growth of fat crystals, change in crystal form, strengthening of network bonds.
Cake	Firmness increases, moist mouthful decreases	Starch retrogradation, moisture migration.
Cheese	Firmness and fracturability increase, springiness decreases	Proteolytic changes.
Chocolate	Graininess develops, surface 'bloom'	Change of crystal form, sugar or fat crystallize on surface.
Fresh fruit	Softening, wilting, loss of crispness, loss of juiciness	Pectin degradation, respiration, bruising, loss of moisture.
Ice cream	Coarseness increases, butteriness, sandiness, crumbliness	Ice crystals enlarge. clumping of fat globules. crystallization of lactose. poor protein hydration.
Milk Powder	Stickiness	Moisture absorption, lactose changes from glassy to crystalline state.

Definitions of Texture

The following definitions were all developed by the International Organization for Standardization (ISO):

- **Texture** is the attribute resulting from a combination of physical properties perceived by the senses of kinesthesia, touch (including mouth, feel, sight and hearing). The properties may include size, shape, number, nature, and conformation of constituent structural elements.

- **Consistency.** ‘All the sensations resulting from stimulation of the mechanical receptors and tactile receptors, especially in the region of the mouth, and varying with the texture of the product.’
- **Hard** (adjective). ‘As a texture characteristic, describes a product which displays substantial resistance to deformation or breaking. The corresponding noun is hardness.’
- **Soft** (adjective). ‘As a texture characteristic, describes a product which displays slight resistance to deformation. The corresponding noun is softness.’
- **Tender** (adjective). ‘As a texture characteristic, describes a product which, during mastication, displays little resistance to breaking. The corresponding noun is tenderness.’
- **Firm** (adjective). ‘As a texture characteristic, describes a product which, during mastication, displays moderate resistance to breaking. The corresponding noun is firmness.’
- **Hardness** (noun) is the perceived force required to break the sample into several pieces during the first bite by the molars.
- **Crunchiness** (noun) is the perceived cumulative intensity of force required by repeated incremental failures of the product by chewing up to five times with the molars.

Texture Profile Analysis (TPA)

Texture profile analysis (TPA) is an empirical technique that compresses a bite-size piece of food two times in a reciprocating motion that imitates the action of the jaw and extracted from the resulting force-time curve a number of textural parameters that correlate well with sensory evaluation of those parameters.

The principle of the TPA test is illustrated in **Figure 4.15**: A ‘bite-size’ sample of food of standard size and shape is placed on the baseplate and **compressed** and **decompressed two times** by a platen attached to the drive system. To imitate the chewing action of the teeth there should be a high compression.

Figure 4.16 shows a typical TPA curve generated by General Foods group (G.F. Texturometer). The height of the force peak on the first compression cycle (first bite) was defined as **hardness**; **Figure 4.16**, **A** is the beginning of the first compression and **B** is the beginning of the second compression. **Fracturability** (originally called brittleness) was defined as the force of the significant break in the curve on the first bite (shown as a dashed line in **Figure 4.16**). The ratio of the positive force areas under the first and second compressions (A_2/A_1) was defined as **cohesiveness**. The negative force area of the first bite (A_3) represented the work necessary to

pull the compressing plunger away from the sample and was defined as **adhesiveness**. The distance that the food recovered its height during the time that elapsed between the end of the first bite and the start of the second bite (BC) was defined as **springiness** (originally called elasticity). Two other parameters were derived by calculation from the measured parameters: **gumminess** was defined as the product of hardness $\times$ cohesiveness; **chewiness** was defined as the product of gumminess $\times$ springiness (which is hardness $\times$ cohesiveness $\times$ springiness).

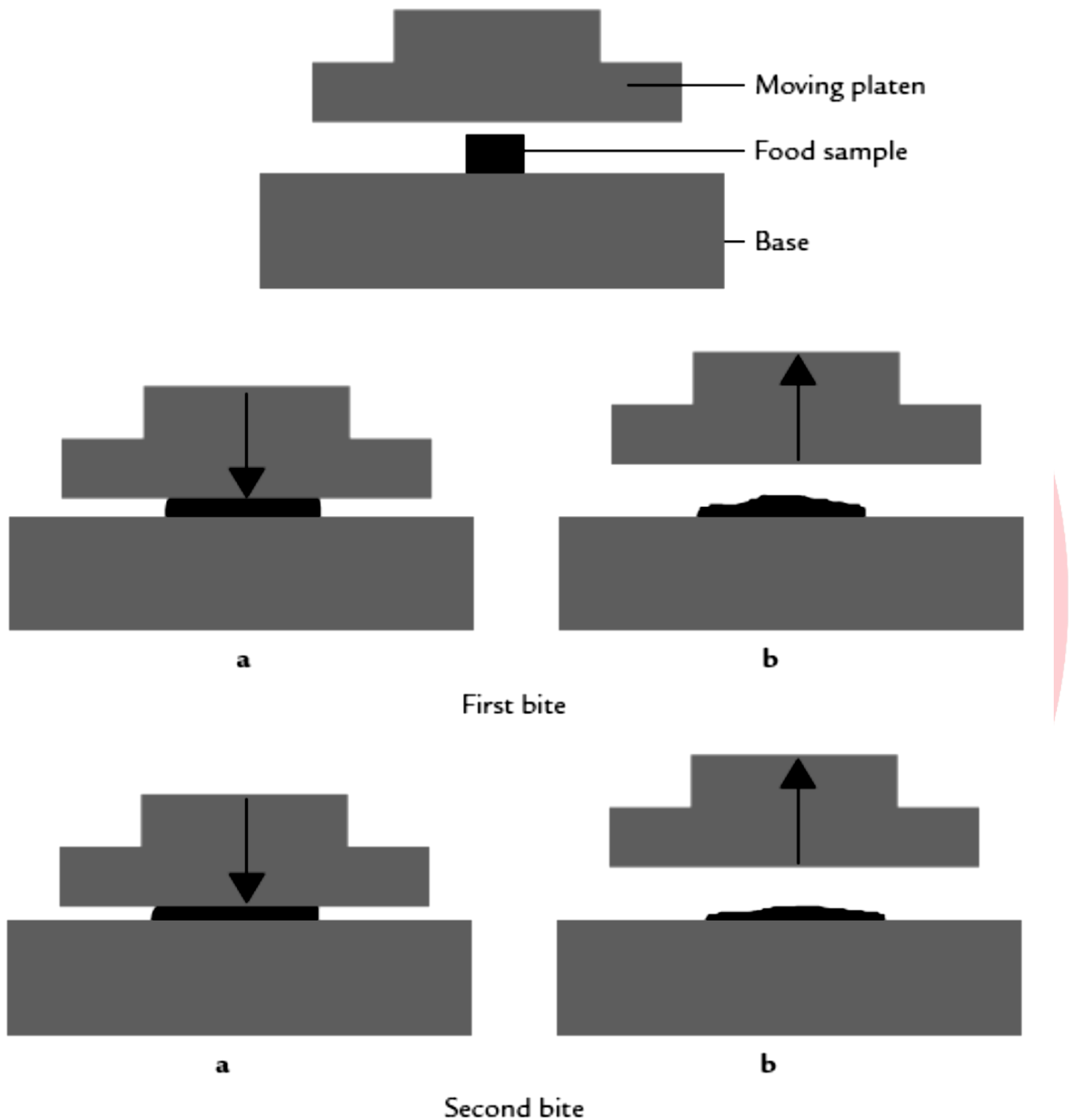


Fig.4.15 Schematic diagram of the two compressions required for the texture profile analysis test. (a) Downstroke actions during the first and second bites; (b) upstroke actions during the first and second bites.

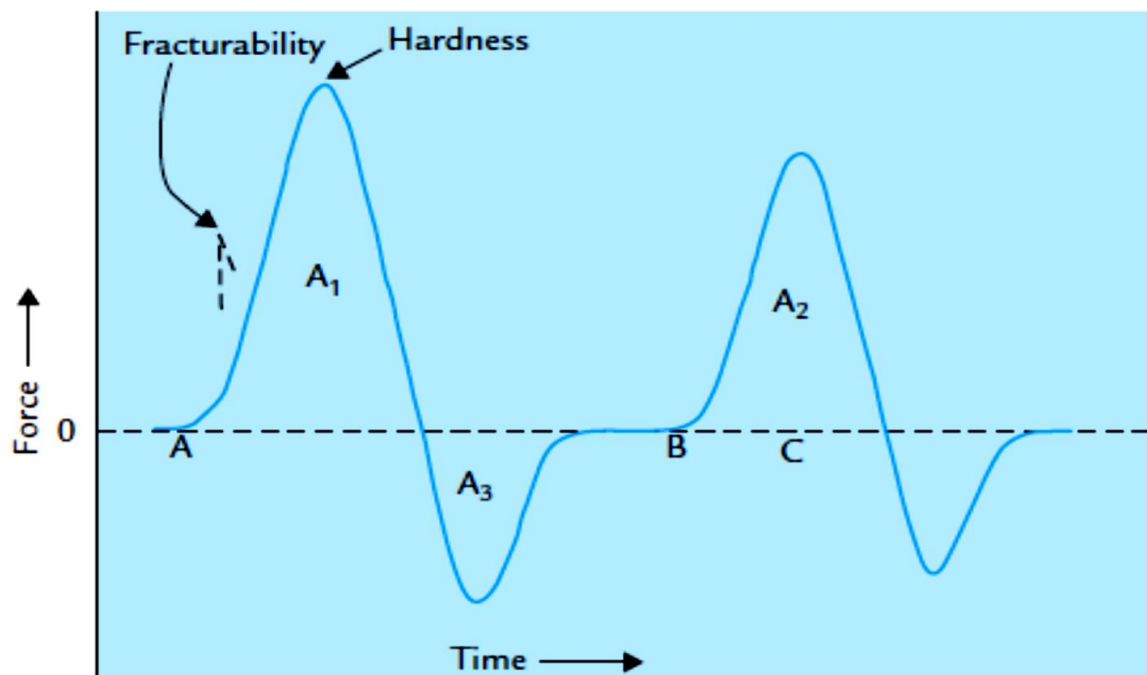


Fig. 4.16 A typical GF Texturometer curve

The Instron, TA.XT2 Texture Analyzer and some other universal testing machines have been adapted to perform a modified texture profile analysis. A typical Instron TPA curve is shown in figure 4.17

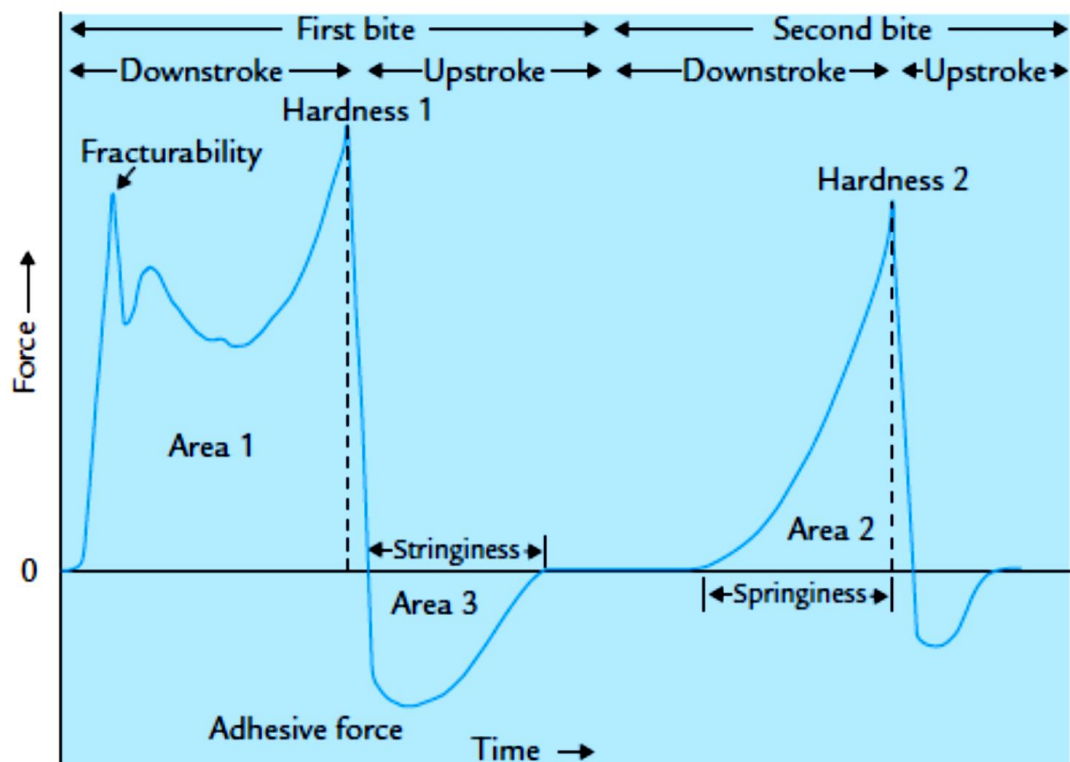


Fig.4.17 A generalized texture profile analysis curve obtained from the Instron Universal Testing Machine.

Color Analysis

Color, flavor, and texture are the three principal quality attributes that determine food acceptance, and color has a far greater influence on our judgment than most of us appreciate. We use color to determine if a banana is at our preferred ripeness level, and a discolored meat product can warn us that the product may be spoiled.

Food scientists who establish **quality control specifications** for their product are very aware of the importance of color and appearance. While subjective visual assessment and use of visual color standards are still used in the food industry, instrumental color measurements are extensively employed. Objective measurement of color is desirable for both research and industrial applications, and the ruggedness, stability, and ease of use of today's color measurement instruments have resulted in their widespread adoption.

Color can be defined as the sensation that is experienced by an individual when radiant energy within the visible spectrum (380-770nm) falls upon the retina of the eye, and a colorant is a pigment that is used to color a product. For the phenomenon of color to occur there must be (1) a colored object, (2) light in the visible region of the spectrum, and (3) an observer. **Colorimetry** is the science of color measurement. It is possible to define color in mathematical units; however, those numbers do not easily relate to the observed color. A number of color-ordering systems and color spaces have been developed that better agree with visual assessment. In food research and quality control, instruments are needed which provide repeatable data that correspond to how the eye sees color.

Color specification systems

There are verbal, visual matching, and instrumental methods for describing and specifying color. Color is three dimensional, and any color-order system will need to address **hue**, what we instinctively think of as color (e.g., red, blue, green), **value**, which represents lightness and darkness, and **chroma** or **saturation** which indicates intensity. When attempting to verbally describe a color defect or problem, one should attempt to use these three qualities in formulating a color description.

Visual systems

The **Munsell** system is probably the best known and most widely used visual color-ordering system. It was developed by A.H. Munsell, a Boston art teacher, in 1905. In this system, Red, Yellow, Green, Blue, and Purple plus five adjacent pairs, Green-Yellow, Yellow-Red, Red-Purple, Purple-Blue, and Blue-Green describe hue. Value is that quality of color described by lightness and darkness, from white to gray to black. Value is designated from zero (absolute black) to ten (absolute white). **Chroma** is that quality that describes the extent a color differs from a gray of the same value. It is designated in increasing numbers starting with 0 (neutral

gray) and extending to/16 or even higher. A change from pink to red is an example of an increase in chroma. In Munsell notation, hue is listed first and designated by a number and letter combination. Numbers run from 1 to 100, and the letters are taken from the ten major hue names, e.g., 10 GY. Value follows with a number from 0 to 10 followed by a slash mark, which is followed by a number for chroma (e.g., 5R 5/10). **Figure 4.18** illustrates the Munsell color system, showing a circle of hues at value 5 chroma 6; the neutral values from 0 to 10; and the chroma of purple-blue (5PB) at value 5. The ten named hues are shown with additional intermediate hues interspersed. The distance from the core to the edge shows increasing chroma.

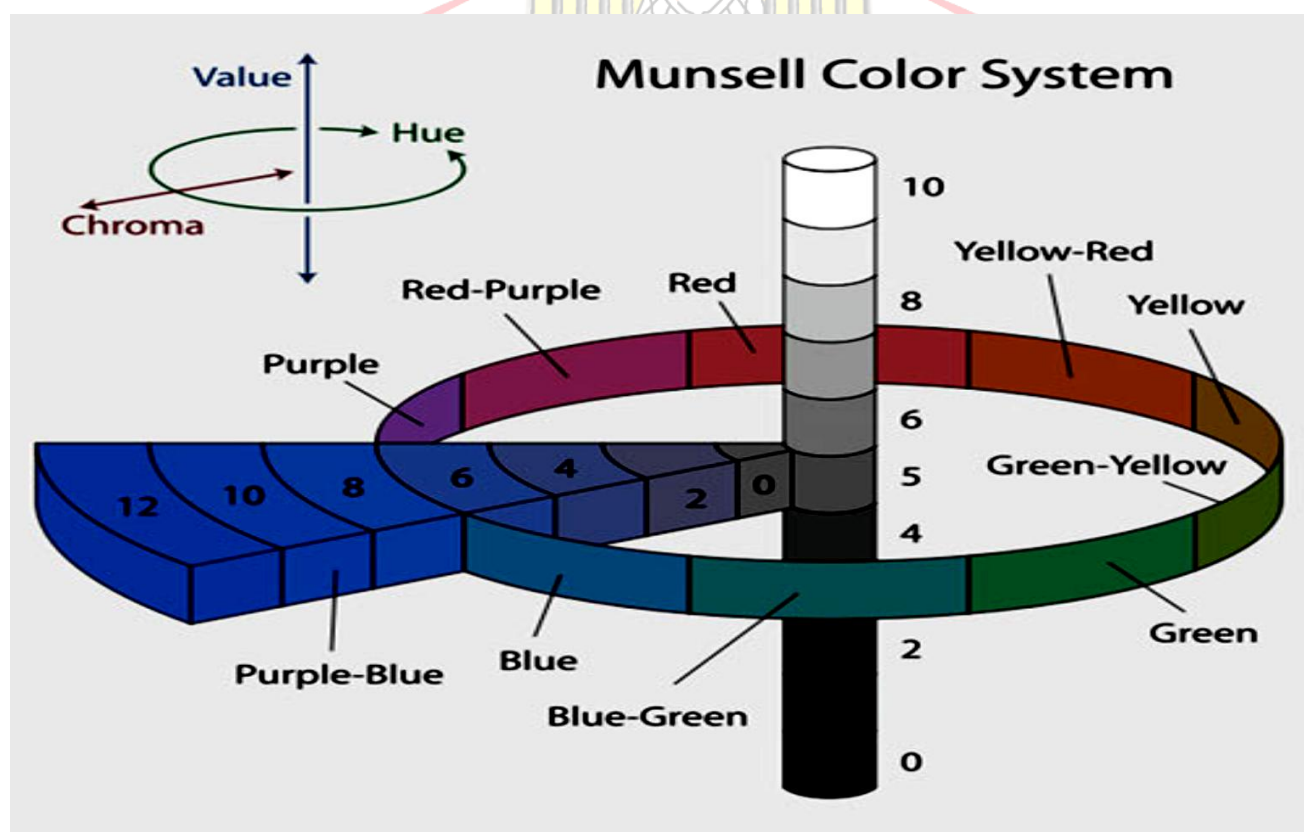


Fig. 4.18 Diagram of the Munsell color system, showing: a circle of hues at value 5 chroma 6; the neutral values from 0 to 10; and the chromas of purple-blue (5PB) at value 5.

Instrumental measurement of color

The CIE Tristimulus System

Early investigators believed that it was possible to measure color since the basic mechanisms for color perception were well understood. It was realized that to objectively measure color there needed to be standardization of light sources. The **CIE (Commission Internationale de l'Eclairage or the International Commission on Illumination)** is the main international organization concerned with color and color measurement. Standard illuminants for color measurement were first established in 1931 by the CIE.

Scientists knew that a color sensation could be matched by mixing three colored lights. The researchers conducted independent experiments in which people with normal color vision visually matched spectral (single wavelength) light by mixing different amounts of three primary lights (red, green, and blue) using rheostats. The process was repeated for test colors covering the entire visible spectrum. The field of view for these experiments which is similar to viewing a dime at an arm's length. The purpose of these viewing conditions was to have primary involvement of the fovea, the retinal area of greatest visual acuity. The red, green, and blue response factors were averaged and mathematically converted to $\bar{x}$, $\bar{y}$, and $\bar{z}$ functions that quantify the red, green, and blue cone sensitivity of the average human observer. The observer functions were standardized and adopted by CIE in 1931 as the CIE 2° Standard Color Observer. The Standard Observer curves provide human sensory response factors that are used in color measurement worldwide (Figure 4.19). Later it was realized that the cones spread beyond the fovea, and more realistic data could be obtained from a larger field of view. The experiment was repeated using a 10° field of view and adopted by CIE in 1964 as the 10° Standard Observer. Both sets of data are used today, but the 10° Standard Observer is preferable because it better correlates to the visual assessments typically made with a larger field of view.

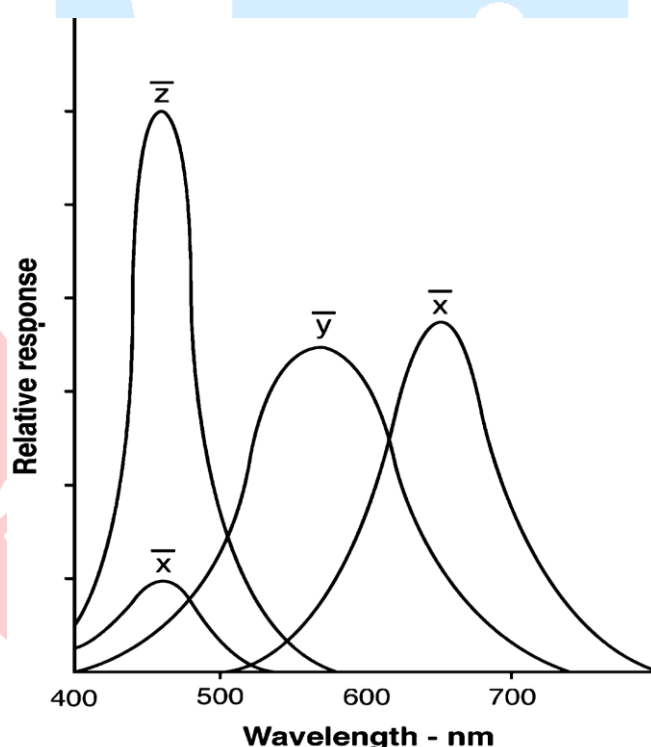


Fig. 4.19 Standard observer curves showing the relationship between the red ($\bar{x}$), blue ($\bar{z}$), and green ($\bar{y}$) cone sensitivity and the visible spectrum.

With the adoption of standard observer functions and standard illuminants, it became possible to convert the spectral transmission or reflectance curve of any object to three numerical values. These numbers are known as the CIE tristimulus values, X, Y, and Z, the amounts of red, green, and blue primaries required to give a color match. The data values for a standard illuminant and the standard observer functions are multiplied by the % reflectance or % transmission values for the object at selected wavelengths. Summation of the products for the wavelengths in the visible spectrum (essentially integrating the areas under the three curves) gives the resulting X, Y, and Z tristimulus values. This can mathematically be represented as follows:

$$X = \int_{380}^{750} RE\bar{x} dx$$

Eq. 4.6

$$Y = \int_{380}^{750} RE\bar{y} dy$$

Eq. 4.7

$$Z = \int_{380}^{750} RE\bar{z} dz$$

Eq. 4.8

Where:

R = sample spectrum

E = source light spectrum

$\bar{x}$, $\bar{y}$, $\bar{z}$ = standard observer curves

With the objective of plotting the three coordinates in two dimensions, the CIE converted the X, Y, and Z tristimulus values to x, y, and z coordinates by the following mathematical operation:

$$x = \frac{X}{X + Y + Z}$$

Eq. 4.9

$$y = \frac{Y}{X + Y + Z}$$

Eq. 4.10

$$z = \frac{Z}{X + Y + Z}$$

Eq. 4.11

Since $x + y + z = 1$, only two coordinates are needed to describe color as $z = 1 - (x + y)$.

Manual calculation of XYZ tristimulus values from reflectance/transmission spectra is a tedious operation. Modern colorimetric spectrophotometers measure the light reflected or transmitted from an object, and the data are sent to a processor where it is multiplied by standard illuminant and standard observer functions to give the XYZ tristimulus values.

Tristimulus Colorimeters and Color Spaces

Richard S. Hunter, Deane B. Judd, and Henry A. Gardner were among the pioneering scientists who in the 1940s were working to develop color-measuring instruments that would overcome the disadvantages of the CIE Spectrophotometric tristimulus system.

The Hunter color solid (Figure 4.20) was first published in 1942 where L indicated lightness, a , the red (+) or green (–) coordinate, and b , the yellow (+) or blue (–) coordinate. The Hunter $L a b$ color space has been widely adopted by the food industry. It is very effective for measuring color differences.

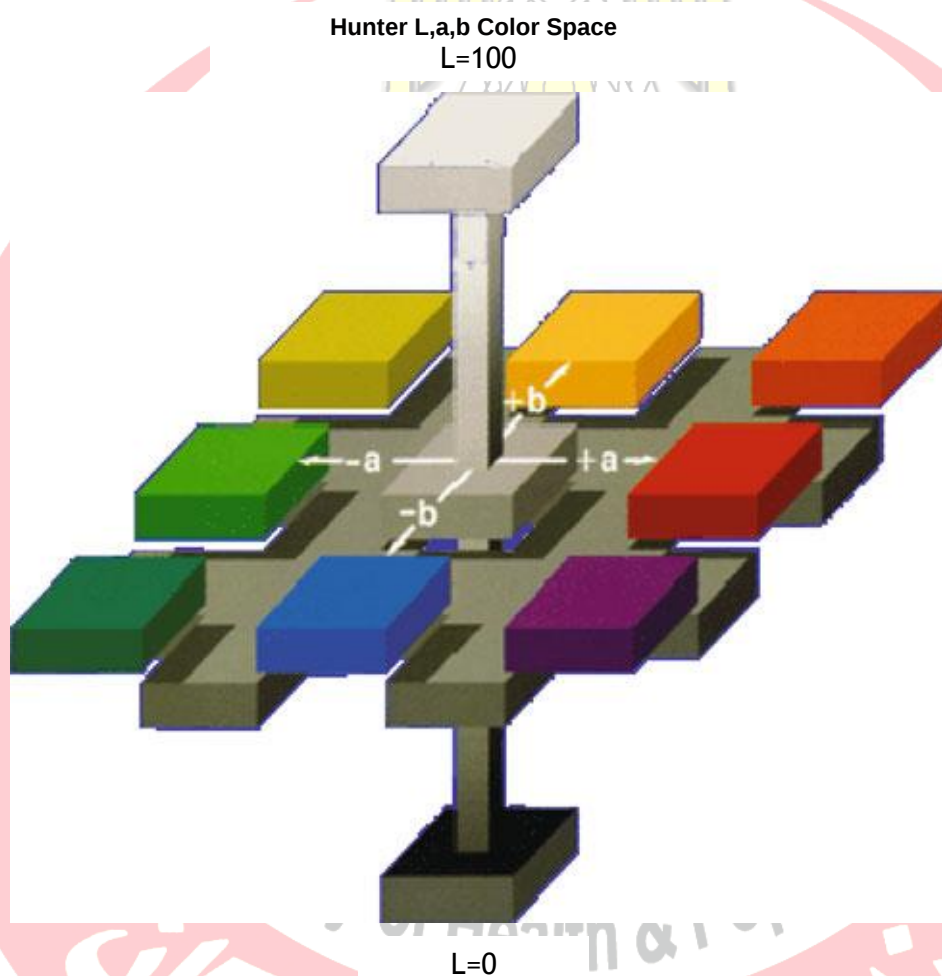


Fig . 4.20 The Hunter L, a, b Color Solid

In 1976, the CIE officially adopted the modified system as **CIELAB** with the parameters $L^*a^*b^*$. L^* indicates lightness (0 to 100) with 0 being black and 100 being white. The coordinate a^* is for red (+) and green (–), and b^* is for yellow (+) and blue (–). The limits for a^* and b^* are approximately + or –60. Figure 4.21 shows a portion of the a^*, b^* chromaticity diagram where a^* and b^* are both positive, representing a color range from red to yellow. Point A is the plot of a^* and b^* for a red apple. The angle from the start of the $+a^*$ axis to point A can be calculated

and is known as **hue angle**, h or H^* , which represents the dominating color. The distance from the center to point A is **chroma**. These parameters calculated as follows:

$$L = 116 (Y/Y_n)^{1/3} - 16 \quad \text{Eq. 4.12}$$

$$a = 500 [(X/X_n)^{1/3} - (Y/Y_n)^{1/3}] \quad \text{Eq. 4.13}$$

$$b = 200 [(Y/Y_n)^{1/3} - (Z/Z_n)^{1/3}] \quad \text{Eq. 4.14}$$

$$\Delta E = (a^2 + b^2 + L^2)^{1/2} \quad \text{Eq. 4.15}$$

The function browning index “BI” which has been proposed as a suitable measure of visual browning was calculated following equation:

$$BI = 100 (x - 0.31) / 0.172 \quad \text{Eq. 4.16}$$

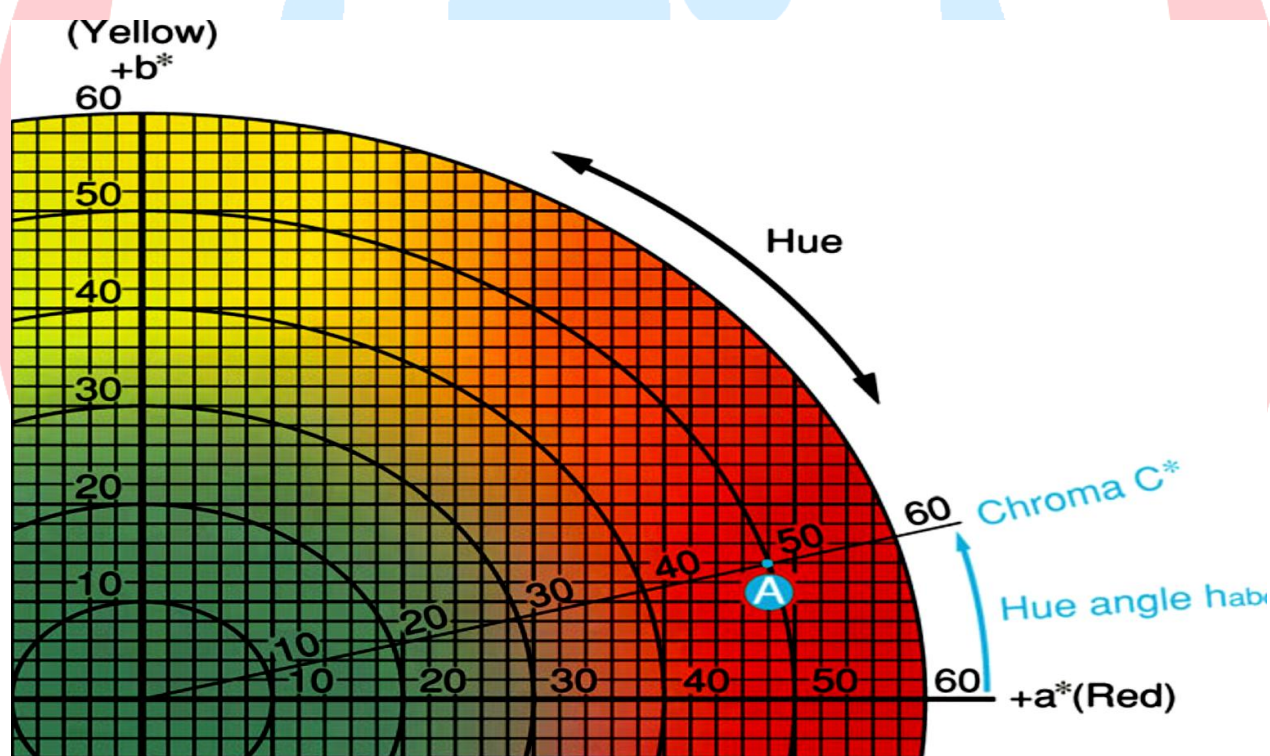
Where:

$$x = X / (X + Y + Z) \quad \text{Eq. 4.17}$$

$$\text{Hue} = \tan^{-1} b / a \quad \text{Eq. 4.18}$$

The colour intensity (chroma) calculated as follows:

$$C = (a^2 + b^2)^{1/2} \quad \text{Eq. 4.19}$$



$$\text{Chroma } C^* = \sqrt{(a^*)^2 + (b^*)^2}$$

$$\text{Hue angle } h_{ab} = \tan^{-1} \left\{ \frac{a^*}{b^*} \right\}$$

Fig. 4.21 A portion of an a^* , b^* chromaticity diagram showing the position A for a red apple.

Interaction of Light with Sample

When a sample is illuminated with light a number of things occur that are illustrated in **Figure 4.22**. Light for which the angle of reflection is equal to the angle of incidence is described as specular light. Smooth polished surfaces will appear glossy because of the high degree of specular reflection. Rough surfaces will have a great deal of diffuse reflection and will have a dull or matte appearance. Selective absorption of light will result in the appearance of color. Opaque samples will reflect light. Transparent samples will primarily transmit light, and translucent samples will both reflect and transmit light. Ideal samples for color measurement will be flat, smooth, uniform, matte, and either opaque or transparent. A brick of colored cheddar cheese is one of the few food examples that come close to having those characteristics.

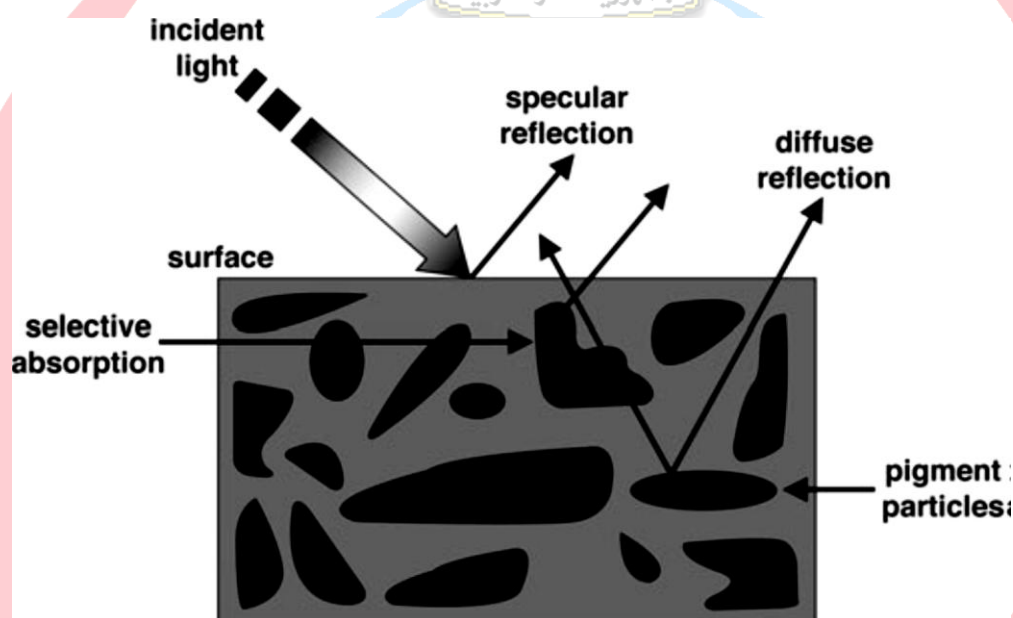


Fig .4.22 Interaction of light with an object

Chapter 5

Compositional Analysis of Foods

Objectives

- After reading this chapter, you should be able to:
 - Recognizes principles of moisture, protein, fat, carbohydrate and vitamins analysis of foods using instruments.
 - Practical application of these methods in food analysis laboratory.

Moisture and Total Solids Analysis

Importance of Moisture Assay

One of the most fundamental and important analytical procedures that can be performed on a food product is an assay for the amount of moisture. The dry matter that remains after moisture removal is commonly referred to as **total solids**. This analytical value is of great economic importance to a food manufacturer because water is an inexpensive filler. The following listing gives some examples in which moisture content is important to the food processor.

1. Moisture is a quality factor in the preservation of some products and affects stability in:

- (a) Dehydrated vegetables and fruits
- (b) Dried milks
- (c) Powdered eggs
- (d) Dehydrated potatoes
- (e) Spices and herbs

2. Moisture is used as a quality factor for:

- (a) Jams and jellies to prevent sugar crystallization
- (b) Sugar syrups
- (c) Prepared cereals - conventional, 4-8%; puffed, 7-8%.

3. Reduced moisture is used for convenience in packaging or shipping of:

- (a) Concentrated milks
- (b) Liquid cane sugar (67% solids) and liquid corn sweetener (80% solids)
- (c) Dehydrated products (these are difficult to package if too high in moisture)
- (d) Concentrated fruit juices

4. Moisture (or solids) content is often specified in compositional standards (i.e., Standards of Identity)

- (a) Cheddar cheese must be $\leq 39\%$ moisture.
- (b) Enriched flour must be $\leq 15\%$ moisture.
- (c) Pineapple juice must have soluble solids of $\geq 10.5^\circ$ Brix (conditions specified).
- (d) Glucose syrup must have $\geq 70\%$ total solids.

5. Food Processing Operations. A knowledge of the moisture content is often necessary to predict the behavior of foods during processing, e.g. mixing, drying, flow through a pipe or packaging.

Moisture Content of Foods

The moisture content of foods varies greatly as shown in Table 5.1. Water is a major constituent of most food products. The approximate, expected moisture content of a food can affect the choice of the method of measurement.

Table 5.1 Moisture Content of Selected Foods

Food	Approximate Percent Moisture (Wet Weight Basis)
White bread enriched (wheat flour)	13.4
Wheat flour, whole-grain	10.3
Corn flakes cereal	3.5
Milk, reduced fat, fluid, 2%	89.3
Yogurt, plain, low fat	85.1
Cottage cheese, low fat or 2% milk fat	80.7
Cheddar cheese	36.8
Beef, raw	73.3
Chicken, broilers and fryers, light meat, meat and skin, raw	68.6
Egg, whole, raw, fresh	75.8
Watermelon, raw	91.5
Apples, raw, with skin	85.6
Raisins	15.3
Potatoes, microwaved, cooked	72.4
Cucumbers	95.2
Sugar granulated	0.0
Sugar, brown	1.3

From US Department of Agriculture, Agricultural Research Service (2009) USDA National Nutrient Database for Standard Reference. Home Page, <http://www.ars.usda.gov/ba/bhnrc/ndl>

Forms of Water in Foods

The ease of water removal from foods depends on how it exists in the food product. The three states of water in food products are:

1. **Free water:** This water retains its physical properties and thus acts as the dispersing agent for colloids and the solvent for salts.
2. **Adsorbed water:** This water is held tightly or is occluded in cell walls or protoplasm and is held tightly to proteins.
3. **Water of hydration:** This water is bound chemically, for example, lactose monohydrate; also, some salts such as $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$.

Sample preparation

Selection of a representative sample and prevent of changes in the properties of the sample prior to analysis are two major potential sources of error in many food analysis procedures. When determining the moisture content of a food it is important to prevent any loss or gain of water. For this reason exposure of a sample to the atmosphere, and excessive temperature fluctuations should be **minimized**. Also, the moisture content of the sample under test must maintain from the time the sample is obtained until all experiments are finished by storing it in a sealed container.

Oven drying method

✚ Principle

In oven drying methods, the sample is heated under specified conditions, and the loss of weight is used to calculate the moisture content of the sample. The amount of moisture determined is highly dependent on the type of oven used, conditions within the oven, and the time and temperature of drying. Various oven methods are approved by AOAC International for determining the amount of moisture in many food products. The methods are simple, and many ovens allow for simultaneous analysis of large numbers of samples. The time required may be from a few minutes to over 24 h.

Moisture removal is sometimes best achieved in a two-stage process. Liquid products (e.g., juices, milk) are commonly predried over a **steam bath** before drying in an oven. Products such as bread and field-dried grain are often air dried, then ground and oven dried, with the moisture content calculated from moisture loss at both air and oven drying steps. Particle size, particle size distribution, sample sizes, and surface area during drying influence the rate and efficiency of moisture removal.

✚ Apparatus

Crucibles (or similar porcelain or metal dishes)
 Drying oven at 101 - 105 °C (or vacuum oven at 68 - 72 °C)
 Glass rods
 Desiccator
 Balance
 Steam bath
 Sand

✚ Procedure

Specific procedures are recommended for individual food products. The following procedures suitable for the range of food types indicated.

1. Composite foodstuffs and fruit and vegetables. Dry a coded, clean crucible (or another suitable container) either over a Bunsen burner for a few minutes or by placing in an oven for about 30 min, cool in a desiccator then weigh. Weigh the crucible along with a small mixing rod. Weigh accurately 4-6 g of the food into the crucible and mix well using the glass rod to aid with the mixing. Record the weight of crucible plus rod plus food. For very wet samples, place on a steam bath until the excess water has evaporated. Dry in an oven at 101-105 °C (or in a vacuum oven at 68 - 72 °C) to **constant weight**, allowing at least 2 h (preferably overnight). Remove and cool in a desiccator. Record the weight of the crucible plus contents after drying and calculate the moisture and total solids content of the food by assuming that the loss in weight of the sample on drying is due to the loss of moisture only. Where overnight drying has not been undertaken, the drying process should be repeated for further 30 min periods until successive weighings differ by less than 0.1 % of the original mass of food sample.

2. Dairy and meat products. The procedure is similar to 1, but uses sand to aid the drying process. Place about 20 g of sand into a coded, clean crucible (or another suitable container) and dry by placing in an oven for about 30 min, cool in a desiccator and weigh along with a small glass rod. Record the weight of crucible plus rod plus sand. Weigh accurately 4-6 g of the food into the crucible and mix using the glass rod. Record the weight of crucible plus rod plus sand plus food.

For very wet samples, e.g. milk, place on a steam bath until the excess water has evaporated. Dry in an air drying oven at 101-105 °C to constant weight, allowing at least 2 h (preferably overnight). Remove and cool in a desiccator. Record the weight of the crucible plus contents after drying and calculate the moisture and total solids content of the food, assuming that the loss in weight of the sample on drying is due to the loss of moisture only.

Where overnight drying has not been undertaken, the drying process should be repeated for further 30 min periods until successive weighings differ by less than 0.1 % of the original mass of food sample.

Calculation

The percentage of moisture is calculated as follows

$$\% \text{ Moisture (wt / wt)} = \frac{W_2 - W_3}{W_2 - W_1} \times 100 \quad \text{Eq. 5.1}$$

Where W_1 = initial weight of empty crucible + rod (+ sand), W_2 = weight of crucible + food + rod (+ sand) before drying, W_3 = final weight of crucible + food + rod (+sand) after drying.

$$\% \text{ Total solids} = 100 - \% \text{ Moisture}$$

Microwave Analyzer

Determination of moisture in food products has traditionally been done using a standard oven, which, though accurate, can take many hours to dry a sample. Other methods have been developed over the years including **infrared** and various types of instruments that utilize halogen lamps or ceramic heating elements. They were often used for “**spot checking**” because of their **speed**, but they lacked the accuracy of the standard oven method.

Microwave moisture analysis, often called **microwave drying**, was the first precise and rapid technique that allowed some segments of the food industry to make in-process adjustment of the moisture content in food products before final packaging. For example, processed cheese could be analyzed and the composition adjusted before the blend was dumped from the cooker. A particular microwave moisture/solids analyzer (CEM Corporation, Matthews, NC), or equivalent, is specified in the AOAC International procedures for total solids analysis of processed tomato products (AOAC, 2007. Method 985.26) and moisture analysis of meat and poultry products (AOAC, 2007. Method 985.14).

There are some considerations when using a microwave analyzer for moisture determination:

- (1) The sample must be of a uniform, appropriate size to provide for complete drying under the conditions specified
- (2) The sample must be centrally located and evenly distributed, so some portions are not burned and other areas are under processed.

- (3) The amount of time used to place an appropriate sample weight between the pads must be minimized to prevent moisture loss or gain before weight determination.

Microwave drying provides a fast, accurate method to analyze many foods for moisture content. The method is sufficiently accurate for routine assay.

The method can't be used with materials that can be **destroyed** at these temperatures or whose **physical composition** can be changed by high heat, such as **sugar**. The samples destruction or composition changes can lead to the samples loss of mass, which will cause moisture-measurement errors.

Another style of microwave oven that includes a **vacuum system** is used in some food plants. This vacuum microwave oven will accommodate one sample in triplicate or three different samples at one time. In 10 min, the results are reported to be similar to 5 h in a vacuum oven at 100° C. The vacuum microwave oven is not nearly as widely used as conventional microwave analyzers, but can be beneficial in some applications.

Infrared Drying

Infrared drying involves penetration of heat into the sample being dried, as compared with heat conductivity and convection with conventional ovens. Such heat penetration to evaporate moisture from the sample can significantly shorten the required drying time to 10-25 min. The infrared lamp used to supply heat to the sample results in a filament temperature of 2000-2500 K (degrees Kelvin). Factors that must be controlled include distance of the infrared source from the dried material and thickness of the sample. The analyst must be careful that the sample does not burn or case harden while drying. Infrared drying ovens may be equipped with forced ventilation to remove moisture air and an analytical balance to read moisture content directly.

No infrared drying moisture analysis techniques are approved by AOAC International currently. However, because of the speed of analysis, this technique is suited for qualitative in-process use.

Rapid Moisture Analyzer Technology

Many rapid moisture/solids analyzers are available to the food industry. In addition to those based on infrared and microwave drying as described previously, compact instruments that depend on high heat are available, such as analyzers that detect moisture levels from 50ppm to 100% using sample weights of 150 mg to 40 g. Using a digital balance, the test sample is placed on an aluminum pan or filter paper and the heat control program (with a heating range of 25-275 °C) elevates the test sample to a constant temperature. As the moisture is driven from

the sample, the instrument automatically weighs and calculates the percentage moisture or solids, (Figure 5.1).

This technology is utilized to cover a wide range of applications within the food industry and offers **quick and accurate** results within minutes. These analyzers are utilized for both production and laboratory use with results **comparable to reference methods**.



IR Moisture/Solids Analyzer



Microwave Moisture/Solids Analyzer

Fig. 5.1 Rapid Moisture Analyzer

Distillation Procedures

Distillation techniques involve codistilling the moisture in a food sample with a **high boiling point solvent** that is immiscible in water, collecting the mixture that distills off, and then measuring the volume of water.

Distillation techniques were originally developed as rapid methods for quality control work, but they are not adaptable to routine testing. The distillation method is an **AOAC-approved** technique for moisture analysis of spices (**AOAC Method 986.21**), cheese (**AOAC Method 969.19**), and animal feeds (**AOAC Method 925.04**). It also can give good **accuracy and precision** for nuts, oils, soaps, and waxes.

Distillation methods cause less thermal decomposition of some foods than oven drying at high temperatures. Adverse chemical reactions are not eliminated but can be minimized by using a solvent with a lower boiling point. This, however, will increase distillation times. Water is

measured directly in the distillation procedure (rather than by weight loss), but reading the volume of water in a receiving tube may be less accurate than using a weight measurement.

Reflux Distillation with Immiscible Solvent

Principle:

Reflux distillation uses either a solvent less dense than water (e.g., toluene, with a boiling point of 110.6°C ; or xylene, with a boiling range of $137\text{--}140^{\circ}\text{C}$) or a solvent more dense than water (e.g., tetrachlorethylene, with a boiling point of 121°C). The advantage of using this last solvent is that material to be dried floats; therefore it will not char or burn. In addition, there is no fire hazard with this solvent.

A Bidwell-Sterling moisture trap (Figure 5.2) is commonly used as part of the apparatus for reflux distillation with a solvent less dense than water.

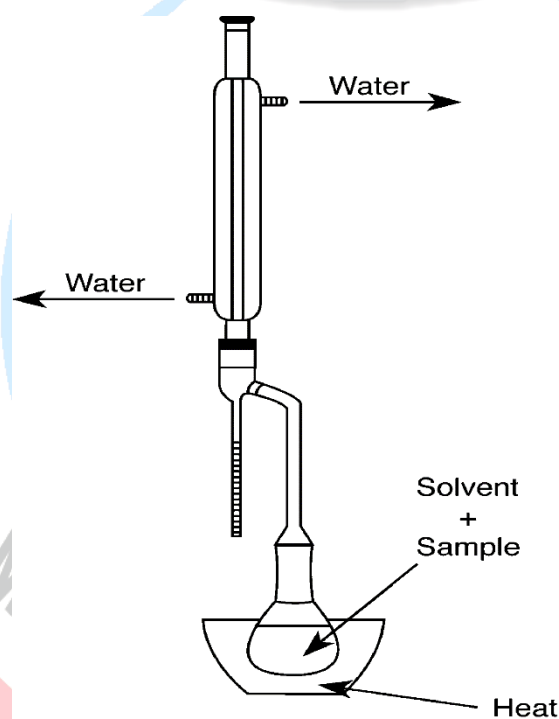


Fig. 5.2 Apparatus for reflux distillation of moisture from a food. This style can be used only where the solvent is less dense than water

Procedure

Place sample in distillation flask and cover completely with solvent.

Fill the receiving tube (e.g., Bidwell-Sterling Trap) with solvent, by pouring it through the top of the condenser.



Bring to a boil and distill slowly at first then at increased rate.



After the distillation has proceeded for approximately 1 hr, use an adapted buret brush to dislodge moisture droplets from the condenser and top part of the Bidwell-Sterling trap.



Slide the brush up the condenser to a point above the vapor condensing area.



Rinse the brush and wire with a small amount of toluene to dislodge adhering water drops.



If water has adhered to the walls of the calibrated tube, invert the brush and use the straight wire to dislodge this water so it collects in the bottom of the tube.



Return the wire to a point above the condensation point, and rinse with another small amount of toluene.



After no more water has distilled from the sample, repeat the brush and wire routine to dislodge adhering water droplets.



Rinse the brush and wire with toluene before removing from the condenser.



Allow the apparatus to cool to ambient temperatures before measuring the volume of water in the trap.



Volume of water x 2 (for a 50 g sample) = % moisture

- When the toluene in the distillation just starts to boil, the analyst will observe a hazy cloud rising in the distillation flask. This is a vaporous emulsion of water in toluene. Condensation occurs as the vapors rise, heating the vessel, the Bidwell-Sterling trap, and the bottom of the condenser. It is also hazy at the cold surface of the condenser, where water droplets are visible. The emulsion inverts and becomes toluene dispersed in water. This turbidity clears very slowly on cooling.

- **Three potential sources of error with distillation should be eliminated if observed:**

1. Formation of emulsions that will not break. Usually, this can be controlled by allowing the apparatus to cool after distillation is completed and before reading the amount of moisture in the trap.
2. Clinging of water droplets to dirty apparatus. Clean glassware is essential, but water seems to cling even with the best cleaning effort. A burette brush, with the handle end flattened so it will pass down the condenser, is needed to dislodge moisture droplets.
3. Decomposition of the sample with a production of water. This is principally due to carbohydrate decomposition to generate water ($C_6H_{12}O_6 \rightarrow 6H_2O + 6C$). If this is a measurable problem, discontinue method use and find an alternative procedure.

Chemical Method: Karl Fischer Titration

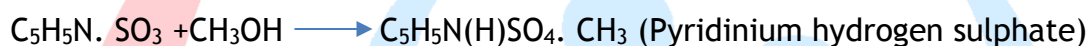
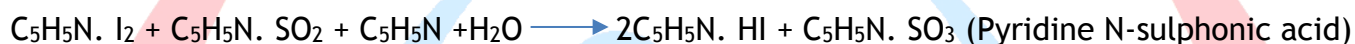
The Karl Fischer titration is particularly adaptable to food products that show erratic results when heated or submitted to a vacuum. This is the method of choice for determination of water in many low moisture foods such as dried fruits and vegetables (AOAC Method 967.19), candies, chocolate (AOAC Method 977.10), roasted coffee, oils and fats (AOAC Method 984.20), or any low-moisture food high in sugar or protein. The method is **quite rapid**, is **accurate**, and uses **no heat**.

Principles

This method is based on the fundamental reaction involving the reduction of iodine by SO₂ in the presence of water:



This was modified to include methanol and pyridine in a four-component system to dissolve the iodine and SO₂:



These reactions show that for each mole of water, 1mol of iodine, 1mol of SO₂, 3 mol of pyridine, and 1mol of methanol are used. For general work, a methanolic solution is used that contains these components in the ratio of 1 iodine: 3 SO₂: 10 pyridine, and at a concentration so that 3.5 mg of water = 1ml of reagent.

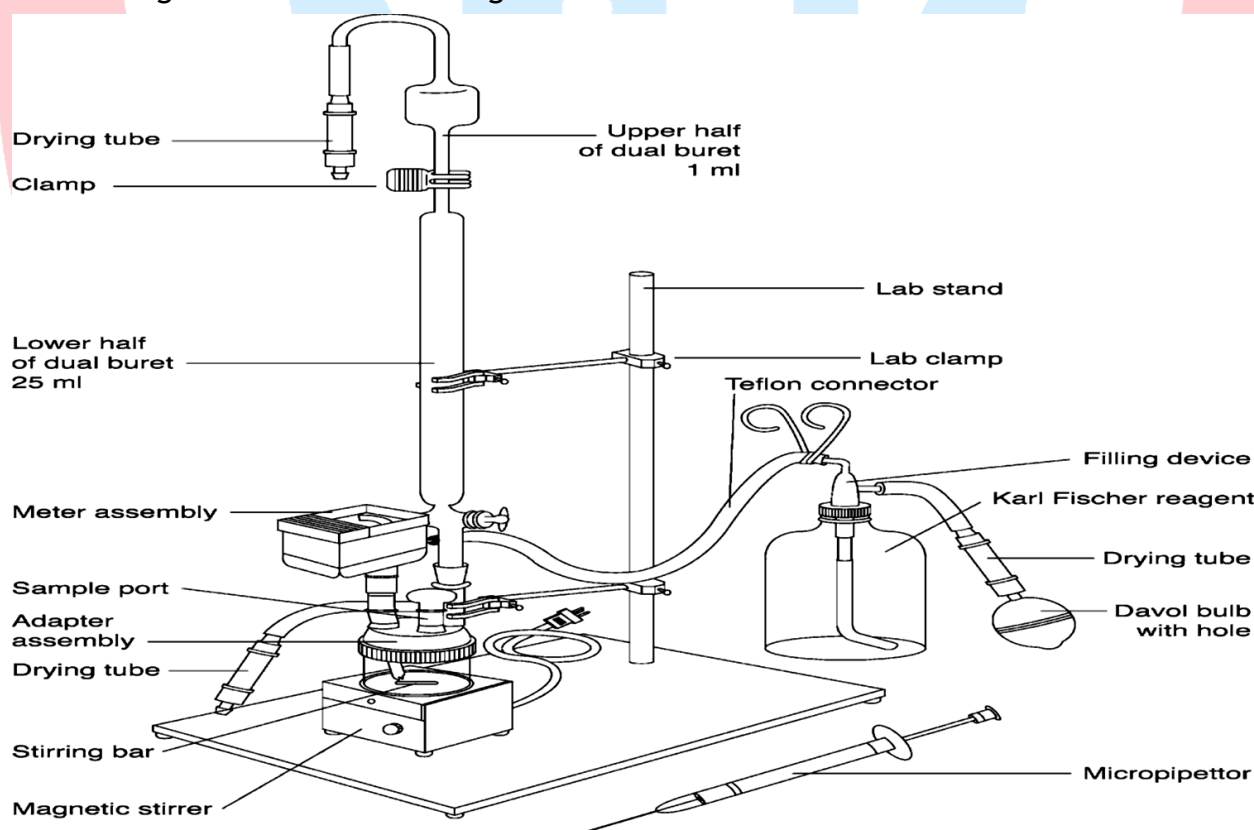


Fig. 5.3 Manual Karl Fischer titration unit



Fig. 5.4 Automated Karl Fischer volumetric titration unit

In a volumetric titration procedure (**Figure 5.3** is manual titration unit; **Figure 5.4** is example of automated titration unit), iodine and SO_2 in the appropriate form are added to the sample in a closed chamber protected from atmospheric moisture. The excess of I_2 that cannot react with the water can be determined visually. The endpoint color is dark red-brown. Some instrumental systems are improved by the inclusion of a potentiometer (i.e., conductometric method) to electronically determine the endpoint, which increases the sensitivity and accuracy. The volumetric titration can be done manually (**Figure 5.3**) or with an automated unit (**Figure 5.4** is one example instrument). The automated volumetric titration units (used for 100 ppm water to very high concentrations) use a pump for mechanical addition of titrant and use the conductometric method for endpoint determination (i.e., detection of excess iodine is by applying a current and measuring the potential).

In a Karl Fischer volumetric titration, the Karl Fischer reagent (**KFR**) is added directly as the titrant if the moisture in the sample is accessible. However, if moisture in a solid sample is inaccessible to the reagent, the moisture is extracted from the food with an appropriate solvent (e.g., methanol). Then the methanol extract is titrated with KFR.

Before the amount of water found in a food sample can be determined, a **KFR water (moisture) equivalence (KFR_{eq})** must be determined. The KFR_{eq} value represents the equivalent amount

of moisture that reacts with 1ml of KFR. Standardization must be checked before each use because the KFR_{eq} will change with time.

The KFR_{eq} can be established with **pure water**, a **water-in-methanol standard**, or **sodium tartrate dihydrate**. Pure water is a difficult standard to use because of inaccuracy in measuring the small amounts required. The water-in-methanol standard is premixed by the manufacturer and generally contains 1mg of water/ml of solution. This standard can change over prolonged storage periods by absorbing atmospheric moisture. Sodium tartrate dehydrate ($\text{Na}_2\text{C}_4\text{H}_4\text{O}_6 \cdot 2\text{H}_2\text{O}$) is a primary standard for determining KFR_{eq}. This compound is very stable, contains 15.66% water under all conditions expected in the laboratory, and is the material of choice to use.

The KFR_{eq} is calculated as follows using sodium tartrate dihydrate:

$$\text{KFR}_{\text{eq}} (\text{mgH}_2\text{O}/\text{ml}) = \frac{36 \text{ gH}_2\text{O}/\text{molNa}_2\text{C}_4\text{H}_4\text{O}_6 \cdot 2\text{H}_2\text{O} \times S \times 1000}{230.08\text{g}/\text{mol} \times A} \quad \text{Eq. 5.2}$$

Where:

KFR_{eq} = Karl Fischer reagent moisture equivalence

S = weight of sodium tartrate dihydrate (g)

A = ml of KFR required for titration of sodium tartrate dehydrate

Once the KFR_{eq} is known, the moisture content of the sample is determined as follows:

$$\% \text{H}_2\text{O} = \frac{\text{KFR}_{\text{eq}} \times K_s}{S} \times 100 \quad \text{Eq. 5.3}$$

where:

KFR_{eq} = Karl Fischer reagent moisture equivalence

K_s = ml of KFR used to titrate sample

S = weight of sample (mg)

Physical Methods

Refractometry

Moisture in liquid sugar products and condensed milks can be determined using a Baumé hydrometer (solids), a Brix hydrometer (sugar content), gravimetric means, or a **refractometer**. If it is performed correctly and no crystalline solids are evident, the refractometer procedure

is rapid and surprisingly accurate (**AOAC Method 9.32.14C**, for solids in syrups). The refractometer has been valuable in determining the soluble solids in fruits and fruit products (**AOAC Method 932.12; 976.20; 983.17**). Principle of refractometer (Chapter 5, Sect. carbohydrate analysis).

Freezing Point

When water is added to a food product, many of the physical constants are altered. Some properties of solutions depend on the number of solute particles as ions or molecules present. These properties are vapor pressure, freezing point, boiling point, and osmotic pressure. Measurement of any of these properties can be used to determine the concentration of solutes in a solution. However, the most commonly practiced assay formilk is the change of the freezing point value. It has economic importance with regard to both raw and pasteurized milk. The freezing point varies within narrow limits, and the vast majority of samples from individual cows fall between -0.503°C and -0.541°C (-0.525 and -0.565°H , temperature in $^{\circ}\text{H}$ or Hortvet, the surname of the inventor of the first freezing point apparatus). The average is very close to -0.517°C (-0.540°H).

The following is used to convert $^{\circ}\text{H}$ to $^{\circ}\text{C}$, or $^{\circ}\text{C}$ to $^{\circ}\text{H}$:

$$^{\circ}\text{C} = 0.9623^{\circ}\text{H} - 0.0024$$

Eq. 5.3

$$^{\circ}\text{H} = 1.03916^{\circ}\text{C} + 0.0025$$

Eq. 5.4

The principal utility of freezing point is to measure for added water. However, the freezing point of milk can be altered by mastitis infection in cows and souring of milk. In special cases, nutrition and environment of the cow, stage of lactation, and processing operations for the milk can affect the freezing point. If the solute remains constant in weight and composition, the change of the freezing point varies inversely with the amount of solvent present. Therefore, we can calculate the percent H_2O added

$$\% \text{H}_2\text{O added} = \frac{0.517 - T}{0.517} \times 100 \quad \text{Eq. 5.5}$$

Where:

0.517 = freezing point in $^{\circ}\text{C}$ of all milk entering a plant

T = freezing point in $^{\circ}\text{C}$ of a sample

The **AOAC Method 961.07** for water added to milk uses a cryoscope to test for freezing points, and assumes a freezing point for normal milk of -0.527°C (-0.550°H). The Food and Drug

Administration will reject all milk with freezing points above -0.503°C (-0.525°H). Since the difference between the freezing points of milk and water is slight and since the freezing point can be used to calculate the **amount of water added**, it is essential that the method be as precise as possible.



Fig. 5.5 A model 4D3 advanced instruments cryoscope for freezing point determination in milk.

Vapor Pressure Methods

A physical parameter that is closely related to the amount of free water present in a food is the **water activity**:

$$A_w = P/P_o$$

Eq. 5.6

Where P is the partial pressure of the water above the food and P_o is the vapor pressure of pure water at the same temperature. Bound water is much less volatile than free water, and therefore the water activity gives a good indication of the amount of free water present. A variety of methods is available for measuring the water activity of a sample based on its vapor pressure. Usually, the sample to be analyzed is placed in a closed container and allowed to come into equilibrium with its environment. The water content in the headspace above the sample is then measured and compared to that of pure water under the same conditions.

Calorimetric Method

Calorimetric techniques such as **differential scanning calorimeter (DSC)** and **differential thermal analysis (DTA)** can be used to measure changes in the heat absorbed or released by a material as its temperature is varied at a controlled rate. The melting point of water depends on its molecular environment: free water normally melts at higher temperature than bound water. Thus by measuring the **enthalpy change** of a sample with temperature, it is possible to obtain an indication of the amounts of water present in different molecular environments.

How to select method of analysis?

A. Understand the **principle of different method**.

B. **Nature of sample**. While most foods will tolerate oven drying at high temperatures, some foods contain volatiles that are lost at such temperatures. Some foods have constituents that undergo chemical reactions at high temperatures to generate or utilize moisture or other compounds, to affect the calculated moisture content. Vacuum oven drying at reduced temperatures may overcome such problems for some foods. However, a distillation technique is necessary for some food to minimize volatilization and decomposition. For foods very low in moisture or high in fats and sugars, Karl Fischer titration is often the method of choice. The use of a pycnometer, hydrometer, and refractometer requires liquid samples, ideally with limited constituents.

Ash Analysis

Ashing is an important first step in proximate or specific mineral analysis. **Ash** refers to the inorganic (mineral) residue remaining after the combustion or complete acid-facilitated oxidation of organic matter in food.

Defintions:

Dry ashing refers to the use of a muffle furnace capable of maintaining temperatures of 500–600 ° C. Water and volatiles are vaporized, and organic substances are burned in the presence of oxygen to form CO₂ and oxides of N₂. In this way, organic materials are removed from the sample. The remaining minerals are converted to oxides, sulfates, phosphates, chlorides, and silicates. Elements such as Fe, Se, Pb, and Hg may partially volatilize with this procedure, so other methods must be used if ashing is a preliminary step for the analysis of these minerals.

Wet ashing is a procedure used to chemically oxidize and remove organic substances using strong acids, oxidizing agents, or combinations thereof. The acids and oxidizing agents used must also be capable of solubilizing the remaining minerals. Hydrochloric, sulfuric, nitric, and perchloric acids are commonly used. Perchloric acid requires a specially designed hood, since it may leave explosive peroxides behind.

Which one do you choose?

Since wet ashing is conducted either at lower temperatures than dry ashing or in sealed vessels, minerals are not lost due to volatility. For this reason, **wet ashing** is preferable to dry ashing as a preparation for specific elemental analysis.

Importance of Ash in Food Analysis

Ashing is the **first step** in preparing a food sample for specific **elemental analysis**, whether for essential nutrients or for highly toxic heavy metals. Because certain foods are high in particular minerals, ash content can be important from nutritional, toxicological, and food quality standpoints. For example, dairy and beef are known to be rich sources of calcium and iron.

Ash Contents in Foods

The ash content of food can range from 0 to 12 %, but rarely exceeds 5 % for fresh foods. The average ash content for various food groups is given in Table [5.2](#).

Table 5.2 Ash content of selected foods

Food	Percent Ash (Wet Weight Basis)
White rice, raw	0.6
Corn meal, whole grain, yellow	1.1
Wheat flour, whole grain	1.6
Macaroni, dry	0.9
Milk, reduced fat, fluid, 2 %	0.7
Evaporated milk, canned	1.6
Butter, with salt	2.1
Cream, fluid, half and half	0.7
Margarine	2.0
Yogurt, plain, low fat	1.1
Bananas, raw	0.8
Apples, raw, with skin	0.2
Raisins	1.9
Potatoes, raw, skin	1.6
Tomatoes, red, ripe, raw	0.5
Eggs, whole, raw, fresh	0.9
Fish fillet, fried	2.5
Chicken, broilers or fryers	1.0
Beef, raw	1.1

From US Department of Agriculture, Agricultural Research Service (2016) USDA National Nutrient Database for Standard Reference. Home Page, <http://ndb.nal.usda.gov/ba/bhnrc/ndl>

Methods of Analysis

Sample Preparation

Ashing does not require large sample sizes. A 2-10 g sample is generally sufficient for ash determination. Some newer instruments allow for sample sizes as low as 250 mg. For this reason, it is necessary to ensure that a **homogenous**, representative sample is obtained. Milling, grinding, and other methods used to homogenize samples will not significantly alter ash values for proximate analysis. In contrast, if ashing is used as a preparatory step for specific mineral analyses, mineral contamination from the environment or from grinding equipment is of potential concern. This can be addressed by soaking crucibles and glassware in an acid bath to solubilize mineral contaminants and rinsing repeatedly with **distilled-deionized water**. The

crucibles themselves can also be “pre-ashed” in a muffle furnace to remove organic contaminants.

Plant Materials

Plant materials are generally oven-dried prior to grinding and ashing. The primary goal for this oven-drying step is to remove moisture. Since samples are often used for multiple determinations (e.g., protein, fiber, lipid), it may be necessary to keep oven temperatures at or below **100 °C** to prevent the destruction or alteration of non-mineral analytes. Plant material with 15 % or less moisture may be ashed without prior drying. If the goal of the analysis is ash or specific mineral analysis alone, low-temperature drying can be accomplished via the use of a muffle furnace equipped with temperature gradient settings. Samples can thus be dried at low temperature and then ashed in the same crucible.

Fat and Sugar Products

Animal products, syrups, and spices require special consideration prior to ashing because of high fat, moisture, or high sugar content that may result in loss of sample due to spattering, swelling, or foaming. Depending on the application, crucible covers or the use of **sealed crucibles** may be used to contain samples. Meats, sugars, and syrups should be evaporated to dryness on a **steam bath or with an infrared (IR) lamp prior to ashing**. For products that tend to form a crust on heating, one or two drops of olive oil (which contains no ash) may be added to allow steam to escape as the crust forms.

A sample may be ashed after drying and fat extraction. In most cases, mineral loss is minimal during drying and fat extraction. Fat-extracted samples should not be heated until flammable extraction solvents (hexane, ether, etc.) have been evaporated completely to avoid the potential risk of solvent ignition or explosion.

Dry Ashing

Principles and Considerations

The method involves incinerating the dry matter at $525 \pm 25^{\circ}\text{C}$ in a **muffle furnace** (Figure 5.6) and weighing the residue obtained.

Crucible selection is critical in ashing because the type depends upon the specific use. Primary considerations relate to chemical stability and resistance to high temperatures. **Porcelain crucibles** are often the crucible of choice.

All crucibles should be marked for identification. Marks on crucibles with a felt-tip marking pen will disappear during ashing in a muffle furnace. Laboratory inks scribed with a steel pin are available commercially.



Fig. 5.6 Muffle furnace, Porcelain crucibles and dry ashing process

Instruments and glassware

Standard laboratory equipment including:

- A. Porcelain crucible measuring 37-42 mm in diameter and 35-50 mm in depth.
- B. Oven set at $102 \pm 2^\circ \text{C}$.
- C. Desiccator with an effective desiccant (e.g. silica gel).
- D. Analytical balance that can weigh to the nearest 0.1 mg.
- E. Crucible tongs.
- F. Muffle furnace set at $525 \pm 25^\circ \text{C}$.

Personal protection

The wearing of heat resistant gloves is recommended when removing the crucibles from the furnace.

Preparation of the crucible

- Heat the crucible in an oven at 102 ± 2 °C for at least 30 min.
- Place the crucible in a desiccator and leave to cool to room temperature.
- Weigh the empty crucible (W_0) to the nearest 0.1 mg using the analytical balance.

Procedures

AOAC International has several dry ashing procedures (e.g., AOAC Methods 900.02 or 920.117, 923.03) for certain individual foodstuffs. **The general procedure includes the following steps:**

1. Weigh a 5- to 10-g sample into a tared crucible (making sure to account for the weight of crucible covers if they are used). Pre-dry if the sample is very moist.
2. Place crucibles in a cool muffle furnace. Use tongs, gloves, and protective eyewear if the muffle furnace is warm.
3. Heat samples for 12-18 h (or overnight) at about 550 °C.
4. Turn off muffle furnace and wait to open it until the temperature has dropped to at least 250 °C, preferably lower. Open the furnace door slowly to avoid losing the powdery ash that may be disturbed by air movement.
5. Using safety tongs, quickly transfer crucibles to a desiccator with a porcelain plate and desiccant. Cover crucibles, close desiccator, and allow crucibles to cool prior to weighing.

$$\% \text{ Ash (dry basis)} = \frac{(W_2 - W_0)}{(W_1 \times \text{DMC})} \times 100 \quad \text{Eq. 5.7}$$

Where:

W_2 = weight after ashing (crucible + Ash)

W_0 = Weight empty crucible

W_1 = original sample weight

DMC = % solids/100

Note: using the dry matter coefficient allows for the direct conversion of wet weight percent ash values to dry weight ash values. For example, if corn meal is 87 % dry matter, the dry matter coefficient would be 0.87. If it is necessary to calculate percent ash on an as received or wet

weight basis (includes moisture), delete the dry matter coefficient from the denominator. If moisture content was determined in the same crucible prior to ashing, the denominator becomes (dry sample wt - empty crucible wt).

Other suggestions that may be helpful and accelerate incineration:

1. High-fat samples should be extracted either by using the crude fat determination procedure or by burning off prior to closing the muffle furnace.
2. Glycerin, alcohol, and hydrogen will accelerate ashing.
3. Samples such as jellies will spatter and can be mixed with cotton wool to avoid this.
4. Salt-rich foods may require a separate ashing of water-insoluble components and salt-rich water extract. Use a crucible cover to prevent spattering.
5. An alcoholic solution of magnesium acetate can be added to accelerate ashing of cereals. An appropriate blank determination is necessary.

Wet Ashing

Principle, Materials, and Applications

Wet ashing is sometimes called **wet oxidation** or **wet digestion**. Its primary use is preparation for specific mineral analysis. Often, analytical testing laboratories use only wet ashing to prepare samples for certain mineral analyses (e.g., Fe, Cu, Zn, P), because losses would occur by volatilization during dry ashing.

There are several **advantages** to using the wet ashing procedure. Minerals will usually stay in solution, and there is little or no loss from volatilization because of the lower temperature. The oxidation time is short and requires a hood, hot plate, and long tongs, plus safety equipment. The **disadvantages** of wet ashing are that it takes virtually constant operator attention, corrosive reagents are necessary, and only small numbers of samples can be handled at any one time. If the wet digestion utilizes perchloric acid, all work needs to be carried out in an expensive special fume hood called a **perchloric acid hood**, since working under a normal fume hood could lead to the deposit of explosive peroxides in the ventilation system.

Unfortunately, using a single acid for wet ashing does not give complete and rapid oxidation of organic material, so a mixture of acids often is used. Combinations of the following acid solutions are used most often: (1) **nitric acid**, (2) **sulfuric acid-hydrogen peroxide**, and (3) **perchloric acid**. The nitric-perchloric combination is generally faster than the sulfuric-nitric procedure. Precautions for use of perchloric acid are found in the AOAC methods under "Safe Handling of Special Chemical Hazards." Cautions must be taken when fatty foods are wet ashed using perchloric acid.

Procedures

The following is a wet ash procedure using concentrated nitric and sulfuric acids (*to be performed in a fume hood*)

1. Accurately weigh a dried, ground 1 g sample in a 125 mL Erlenmeyer flask (previously acid washed and dried).
2. Prepare a blank of 3 mL of H_2SO_4 and 5 mL of HNO_3 , to be treated like the samples. (Blank is to be run with every set of samples.)
3. Add 3 mL of H_2SO_4 followed by 5 mL of HNO_3 to the sample in the flask.
4. Heat the sample on a hot plate at 200°C . Brown-yellow fumes will be observed.
5. Once the brown-yellow fumes cease and white fumes from decomposing H_2SO_4 are observed, the sample will become darker. Remove the flask from the hot plate. Do not allow the flask to cool to room temperature.
6. **Slowly** add 3-5 mL of HNO_3 .
7. Put the flask back on the hot plate and allow the HNO_3 to boil off. Proceed to the next step when all the HNO_3 is removed and the color is clear to straw yellow. If the solution is still dark in color, add another 3-5 mL of HNO_3 and boil. Repeat the process until the solution is clear to straw yellow.
8. While on the hot plate, reduce the volume appropriately to allow for ease of final transfer. Allow the sample to cool to room temperature, and then quantitatively transfer the sample to an appropriately sized volumetric flask.
9. Dilute the sample to volume with ultrapure water, and mix well. Dilute further, as appropriate, for the specific type of mineral being analyzed.

Microwave Ashing

Both **wet ashing** and **dry ashing** can be done using microwave instrumentation, rather than the conventional dry ashing in a muffle furnace and wet ashing in a flask or beaker on a hot plate. While the ashing procedures by conventional means can take many hours, the use of **microwave instrumentation** can reduce sample preparation time to minutes, allowing laboratories to increase their sample throughput significantly (5-24 samples per hour). This **advantage** has led to widespread use of microwave ashing, especially for wet ashing, within both analytical and quality control laboratories in food companies.

A. Microwave Wet Ashing

Microwave wet ashing (acid digestion) may be performed safely in either an open- or closed-vessel microwave system. Choice of the system depends on the amount of sample and the temperatures required for digesting. Due to the ability of the closed vessels to withstand higher pressures (some vessels can handle up to 1,500 psi), acids may be heated past their boiling points. This ensures a more complete dissolution of hard-to-digest substances. It also allows the chemist to use nitric acid with samples that might normally require a harsher acid, such as sulfuric or perchloric.

In closed vessels specifically designed for high-temperature/ high-pressure reactions, nitric acid can reach a temperature of 240 °C. Thus, nitric acid is often the acid of choice.

Closed vessel microwave digestion systems (**Figure 5.7 A**) can process up to 40 samples at a time. This system allow the input of time, temperature, and pressure parameters in a step-by-step format (ramping).

Typically, in a closed-vessel microwave system, samples are placed in vessels with the appropriate amount of acid. The vessels are sealed and set on a carousel where the temperature and pressure sensors are connected to a control vessel. The carousel then is placed in the microwave cavity and the sensors connected to the instrument. Time, temperature, pressure, and power parameters are chosen and the unit is started. Digestions normally take less than 30 min. Because of the pressure generated by raising the temperature of a reaction, vessels must be allowed to cool before being opened. (Note that some closed-vessel microwave digestion systems may also be used for acid concentration, solvent extraction and protein hydrolysis with the proper accessories).

Open-vessel digestion systems (Figure 5.7 B) are used often for larger sample sizes (up to 10 g) and for samples that generate substantial amounts of gas as they are digested. Acid (reagent) is automatically added according to the programmed parameters. Sulfuric, nitric, hydrochloric, and hydrofluoric acids, as well as hydrogen peroxide, can all be used in open-vessel systems. These instruments do not require the use of a fume hood, because a vapor containment system contains and neutralizes harmful fumes.

Generally, in an open-vessel microwave system, sample is placed in a vessel and the vessel set in a slot in the microwave system. Time, temperature, and reagent addition parameters are then chosen. The unit is started, the acid is added, and the vapor containment system neutralizes the fumes from the reaction. Samples are typically processed much faster and more reproducibly than on a conventional hot plate.



Fig. 5.7 Microwave digestion systems. (A) closed vessel systems, (B) open vessel systems

B. Microwave Dry Ashing

Compared to conventional dry ashing in a muffle furnace that often takes many hours, microwave muffle furnaces (Figure 5.8) can ash samples in minutes, decreasing analysis time by as much as 97 %. Microwave muffle furnaces can reach temperatures of up to 1200 °C. These systems may be programmed with various methods to automatically warm up and cool down. In addition, they are equipped with exhaust systems that circulate the air in the cavity to help decrease ashing times. Typically, in microwave dry ashing, a desiccated crucible is weighed, sample is added, and the crucible is weighed again. The crucible then is placed in the microwave furnace, and the time and temperature parameters are set. The system is started and the program is run to completion. The crucible then is carefully removed with tongs and reweighed.



Fig. 5.8 Microwave muffle furnace

Fat Analysis

Fats play an essential role in food:

- From a **nutritional** point of view, fats provide energy (37.7 kJ/ g), essential fatty acids (linoleic acid, α -linoleic acid), fat-soluble vitamins (A, D, E, K), phytosterols and antioxidants.
- From a **sensory** point of view, fats contribute to food texture and palatability as well as acting as flavour carriers and precursors of aromatic molecules. The melting and crystallization properties of fats, which depend on chain length and the unsaturation of fatty acid residues as well as the structure of triacylglycerols, significantly affect the sensory perception of foods. The main problem with lipids is that they oxidize easily. This is one of the main causes of food spoilage since, in most cases; the production of compounds with unpleasant flavors or odors (rancidity) is undesirable. Moreover, the oxidation of lipids can reduce the nutritional quality, change the texture, color of food, and produce compounds unsuitable for human consumption. In powders, the lipid substrates mostly affected by oxidation are fractions exposed to air, in the form of free fat.

General Classification

The general classification of lipids that follows is useful to differentiate lipids in foods:

A. Simple Lipids

- **Fats:** Esters of fatty acids with glycerol - triacylglycerols
- **Waxes:** Esters of fatty acids with long-chain alcohols other than glycerols (e.g., vitamin A esters, and vitamin D esters)

B. Compound Lipids

- **Phospholipids:** Glycerol esters of fatty acids, phosphoric acids, and other groups containing nitrogen (e.g., phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, and phosphatidyl inositol)
- **Cerebrosides:** Compounds containing fatty acids, a carbohydrate, and a nitrogen moiety (e.g., galactocerebroside and glucocerebroside)
- **Sphingolipids:** Compounds containing fatty acids, a nitrogen moiety, and phosphoryl group (e.g., sphingomyelins)

C. Derived Lipids

Derived lipids are substances derived from neutral lipids or compound lipids. They have the general properties of lipids - examples are fatty acids, longchain alcohols, sterols, fat-soluble vitamins, and hydrocarbons.

Contents of Lipids in Foods

Foods may contain any or all types of the lipid compounds previously mentioned. The lipid content in bovine milk (Table 5.3) illustrates the complexity and variability of lipids in a food system, having lipids that differ in polarity and concentrations.

Foods contain many types of lipids, but those which tend to be of greatest importance are the triacylglycerols and the phospholipids. **Liquid triacylglycerols** at room temperature are referred to as **oils**, such as soybean oil and olive oil, and are generally of plant origin. **Solid triacylglycerols** at room temperature are termed as **fats**. Lard and tallow are examples of fats, which are generally from animals. The term **fat** is applicable to all triacylglycerols whether they are normally solid or liquid at ambient temperatures. Table 5.4 shows the wide range of lipid content in different foods.

Table 5.3 Lipids of Bovine Milk

Kinds of Lipids	Percent of Total Lipids
Triacylglycerols	97-99
Diacylglycerols	0.28-0.59
Monoacylglycerols	0.016-0.038
Phospholipids	0.2-1.0
Sterols	0.25-0.40
Squalene	Trace
Free fatty acids	0.10-0.44
Waxes	Trace
Vitamin A	(7-8.5 µg/g)
Carotenoids	(8-10 µg/g)
Vitamin D	Trace
Vitamin E	(2-5 µg/g)
Vitamin K	Trace

Adapted from Dairy Chemistry. Jenness R. and Patton S. (1959), John Wiley & Sons, Inc with permission.

General Considerations

By definition, lipids are soluble in organic solvents and insoluble in water. Therefore, water insolubility is the essential analytical property used as the basis for the separation of lipids from proteins, water, and carbohydrates in foods. Glycolipids are soluble in alcohols and have a low solubility in hexane. In contrast, triacylglycerols are soluble in hexane and petroleum ether, which are nonpolar solvents. The wide range of relative hydrophobicity of different lipids makes the selection of a single universal solvent impossible for lipid extraction of foods. Some lipids in foods are components of complex lipoproteins and liposaccharides; therefore, successful

extraction requires that bonds between lipids and proteins or carbohydrates be broken so that the lipids can be freed and solubilized in the extracting organic solvents.

Table 5.4 Fat Content of Selected Foods

Food	Percent Fat (Wet Weight Basis)
White rice, raw	0.7
Sorghum	3.3
Wheat, soft white	2.0
Cracked-wheat bread	3.9
Macaroni, dry	1.5
Milk, reduced fat, fluid, 2%	2.0
Skim milk, fluid	0.2
Cheddar cheese	33.1
Lard, shortening, oils	100.0
Butter, with salt	81.1
Margarine	80.5
Mayonnaise, soybean oil, with salt	79.4
Apples, raw, with skin	0.2
Oranges, raw, all commercial varieties	0.1
Chicken, broilers or fryers	1.2
Eggs, whole, raw, fresh	10.0
Almonds, dried, un-blanchd, dry roasted	52.8
Walnuts, black, dried	56.6

Solvent Extraction Methods

- The total lipid content of a food is commonly determined by organic solvent extraction methods or by alkaline or acid hydrolysis followed by **Mojonnier extraction**.
- For multicomponent food products, acid hydrolysis is often the method of choice.
- Both acid hydrolysis and alkaline hydrolysis methods can be performed using Mojonnier extraction equipment.
- The accuracy of direct solvent extraction methods (i.e., without prior acid or alkaline hydrolysis) greatly depends on the solubility of the lipids in the solvent used and the ability to separate the lipids from complexes.

Sample Preparation

The sample preparation for lipid analysis depends on the type of food and the type and nature of lipids in the food. The extraction method for lipids in liquid milk is generally different from that for lipids in solid soybeans. To analyze the lipids in foods effectively, knowledge of the structure, the chemistry, and the occurrence of the principal lipid classes and their constituents is necessary. Therefore, there is no single standard method for the extraction of all kinds of lipids in different foods. For the best results, sample preparation should be carried out under an inert atmosphere of nitrogen at low temperature to minimize chemical reactions such as lipid oxidation.

Pre-drying sample

Drying the sample at elevated temperatures is undesirable because some lipids become bound to proteins and carbohydrates, and bound lipids are not easily extracted with organic solvents. Vacuum oven drying at low temperature or lyophilization increases the surface area of the sample for better lipid extraction. Pre-drying makes the sample easier to grind for better extraction breaks fat-water emulsions to make fats dissolve easily in the organic solvent, and helps to free fat from the tissues of foods.

Particle Size Reduction

The extraction efficiency of lipids from dried foods depends on particle size; therefore, adequate grinding is very important. The classical method of determining fat in oilseeds involves the extraction of the ground seeds with selected solvent after repeated grinding at low temperature to minimize lipid oxidation. For better extraction, the sample and solvent are mixed in a high-speed device such as a blender.

Acid Hydrolysis

A significant portion of the lipids in foods such as dairy, bread, flour, and animal products is bound to proteins and carbohydrates, and direct extraction with nonpolar solvents is inefficient. Such foods must be prepared for lipid extraction by acid hydrolysis.

Acid hydrolysis can break both covalently and ionically bound lipids into easily extractable lipid forms. The sample can be predigested by refluxing for 1 h with 3N hydrochloric acid. Ethanol and solid hexametaphosphate may be added to facilitate separation of lipids from other components before food lipids are extracted with solvents. For example, the acid hydrolysis of two eggs requires 10ml of HCl and heating in a water bath at 65°C for 15-25 min or until the solution is clear.

Continuous Solvent Extraction Method

Goldfish Method

Principle

Fat is extracted, continuously, with an organic solvent. Solvent is heated and volatilized, then is condensed above the sample. Solvent continuously drips through the sample to extract the fat. Fat content is measured by weight loss of sample or weight of fat removed.



Fig. 5.9 Goldfish fat extractor

Chemicals

Reagent	Hazards
Petroleum ether	Harmful, highly flammable, dangerous for environment
(or Ethyl ether)	Harmful, extremely flammable

Hazards, Precautions, and Waste Disposal

Petroleum ether and ethyl ether are fire hazards; avoid open flames, breathing vapors, and contact with skin. Ether is extremely flammable, is hygroscopic, and may form explosive peroxides. Otherwise, adhere to normal laboratory safety procedures. Wear gloves and safety glasses at all times. Petroleum ether and ether liquid wastes must be disposed of in designated hazardous waste receptacles.

Procedure

1. Weigh predried porous ceramic extraction thimble. Place vacuum oven dried sample in thimble and weigh again. (Sample could instead be combined with sand in thimble and then dried.)
2. Weigh pre-dried extraction beaker.
3. Place ceramic extraction thimble into glass holding tube and then up into condenser of apparatus.
4. Place anhydrous ethyl ether (or petroleum ether) in extraction beaker and put beaker on heater of apparatus.
5. Extract for 4 h.
6. Lower the heater and let sample cool.
7. Remove the extraction beaker and let air dry overnight, then at 100°C for 30min. Cool beaker in desiccator and weigh.

Calculations

Weight of fat in sample= (beaker+fat) -beaker Eq. 5.8

% Fat on dry weight basis

= (g of fat in sample/g of dried sample) × 100 Eq. 5.9

Semi-continuous Solvent Extraction Method

Soxhlet Method

The Soxhlet method (AOAC Method 920.39C for Cereal Fat; AOAC Method 960.39 for Meat Fat) is an example of the semi-continuous extraction method and is described below.

Principle of Method

Fat is extracted, semicontinuously, with an organic solvent. Solvent is heated and volatilized, then is condensed above the sample. Solvent drips onto the sample and soaks it to extract the fat. At 15-20 min intervals, the solvent is siphoned to the heating flask, to start the process again. Fat content is measured by weight loss of sample or weight of fat removed.

This method requires more time than the continuous method. Instrumentation for a more rapid and automated version of the Soxhlet method is available.

Preparation of Sample

If the sample contains more than 10% H₂O, dry the sample to constant weight at 95-100 °C under pressure ≤ 100 mmHg for about 5 h (AOAC Method 934.01).

Procedure

1. Weigh, to the nearest mg, about 2 g of pre-dried sample into a predried extraction thimble, with porosity permitting a rapid flow of ethyl ether. Cover sample in thimble with glass wool.
2. Weigh pre-dried boiling flask.
3. Put anhydrous ether in boiling flask. Petroleum ether may be used instead of anhydrous ether (AOAC Method 960.39).
4. Assemble boiling flask, Soxhlet flask, and condenser.
5. Extract in a Soxhlet extractor at a rate of five or six drops per second by condensation for about 4 h, or for 16 h at a rate of two or three drops per second by heating solvent in boiling flask.
6. Dry boiling flask with extracted fat in an air oven at 100 °C for 30min, cool in desiccator, and weigh.

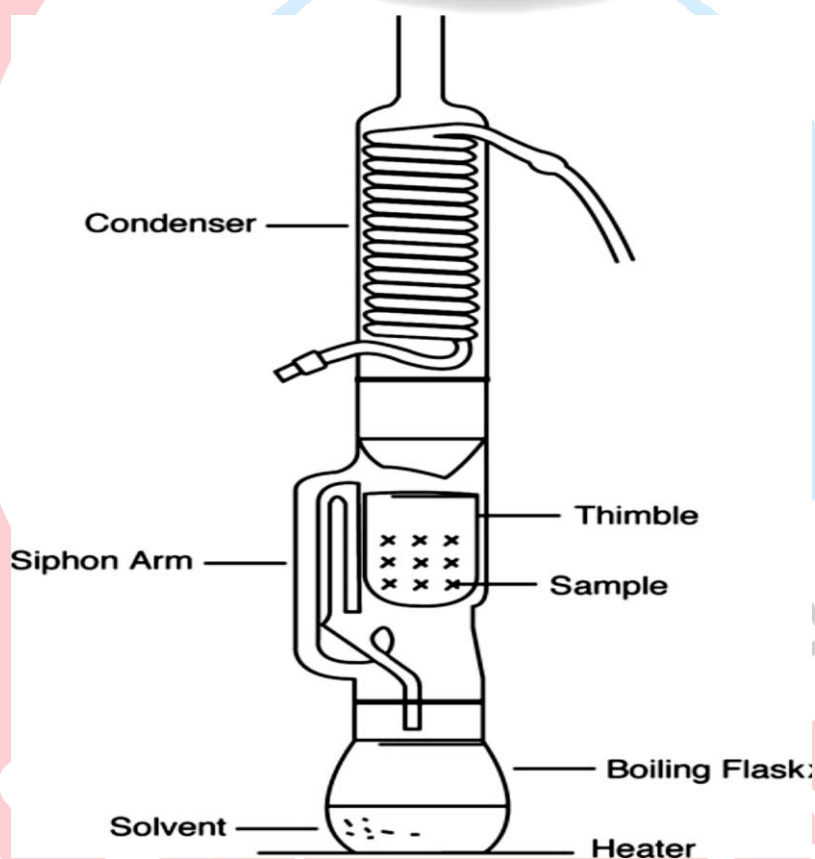


Fig. 5.10 Soxhlet extraction apparatus

Calculations

$$\% \text{ Fat on dry weight basis} = (\text{Weight (g) fat} / \text{Weigh (g) pre-dried sample}) \times 100 \quad \text{Eq. 5.10}$$

Discontinuous Solvent Extraction Methods

Mojonnier Method

Principle of Method: Fat is extracted with a mixture of ethyl ether and petroleum ether. The extract containing the fat is dried and expressed as percent fat by weight.

The Mojonnier test is an example of the discontinuous solvent extraction method and does not require removal of moisture from the sample. It can be applied to both liquid and solid samples. If petroleum ether is used to purify the extracted fat, this method is very similar to the **Roesse-Gottlieb Method (AOAC Method 905.02)** in both principle and practice. The Mojonnier flasks (**Figure 5.11**) are used not only for the Mojonnier and Roesse-Gottlieb methods, but also to do the hydrolysis (acid, alkaline, or combination) prior to fat extraction and GC analysis to determine fat content and fatty acid profile.

The Mojonnier method is applied primarily to dairy foods. The assay uses not only ethyl ether and petroleum ether, but also ammonia and ethanol. Ammonia dissolves the casein and neutralizes the acidity of the product to reduce its viscosity. Ethanol prevents gelation of the milk and ether, and aids in the separation of the ether-water phase. Ethyl ether and petroleum ether serve as lipid solvents, and petroleum ether decreases the solubility of water in the ether phase.

Chemicals

Reagent	Hazards
Ammonium hydroxide	Corrosive, dangerous for the environment
Ethanol	Highly flammable
Petroleum ether	Harmful, highly flammable, dangerous for environment
(or Ethyl ether)	Harmful, extremely flammable

Hazards, Precautions, and Waste Disposal

Ethanol, ethyl ether, and petroleum ether are fire hazards; avoid open flames, breathing vapors, and contact with skin. Ether is extremely flammable, is hygroscopic, and may form explosive peroxides. Ammonia is a corrosive; avoid contact and breathing vapors. Otherwise, adhere to normal laboratory safety procedures. Wear gloves and safety glasses at all times. Petroleum ether and ether liquid wastes must be disposed of in designated hazardous waste receptacles. The aqueous waste can go down the drain with a water rinse.

Equipment

- Analytical balance
- Hot plate
- Mojonnier apparatus (with centrifuge, vacuum oven, and cooling desiccator)

Procedure: Milk Fat Method (AOAC Method 989.05)

1. **Preparation of Sample.** Bring the sample to about 20 °C; mix to prepare a homogeneous sample by pouring back and forth between clean beakers. Promptly weigh or measure the test portion. If lumps of cream do not disperse, warm the sample in a water bath to about 38 °C and keep mixing until it is homogeneous, cool warmed samples to about 20 °C before transferring the test portion.

2. Procedure

- Weigh, to the nearest 0.1mg, 10 g of milk into a Mojonnier fat extraction flask (**Figure 5.11**).
- Add 1.5 ml of NH₄OH (Ammonium Hydroxide) and shake vigorously. Add 2 ml if the sample is sour. NH₄OH neutralizes the acidic sample and dissolves protein.
- Add 10ml of 95% ethanol and shake for 90 s. The alcohol prevents possible gel formation.
- Add 25 ml of ethyl ether and shake for 90 s. The ether dissolves the lipid.
- Cool if necessary, and add 25 ml of petroleum ether and shake for 90 s. The petroleum ether removes moisture from the ethyl ether extract and dissolves more nonpolar lipid.
- Centrifuge for 30 s at 600 rpm.
- Decant ether solution from the Mojonnier flask into the previously weighed Mojonnier fat dish.
- Perform second and third extractions in the same manner as for the first extraction described previously (ethanol, ethyl ether, petroleum ether, centrifugation, decant).
- Evaporate the solvent in the dish on the electric hot plate at ≤100 °C in a hood.
- Dry the dish and fat to a constant weight in a forced air oven at 100 °C ± 1 °C.
- Cool the dish to room temperature and weigh.

Calculations

$$\% \text{ Fat} = \frac{\{[(\text{wt dish} + \text{fat}) - (\text{wt dish})] - (\text{avg wt blank residue})\}}{\text{Wt. sample}} \times 100 \quad \text{Eq. 5.11}$$



Fig. 5.11 Mojonnier fat extraction flasks

Non-solvent Wet Extraction Methods

Babcock Method for Milk Fat (AOAC Method 989.04 and 989.10)

Principle. In the Babcock method, sulfuric acid H_2SO_4 is added to a known amount of milk in the Babcock bottle. The sulfuric acid digests protein, generates heat, and releases the fat. Centrifugation and hot water addition isolate fat for quantification in the graduated portion of the test bottle. The fat is measured volumetrically, but the result is expressed as percent fat by weight.

Hazards, Precautions, and Waste Disposal

Concentrated sulfuric acid is extremely corrosive; avoid contact with skin and clothes and breathing vapors. Wear gloves and safety glasses at all times. Otherwise, adhere to normal laboratory safety procedures. Sulfuric acid waste must be disposed of in a designated hazardous waste receptacle.

For safety and accuracy reasons, dispense the concentrated sulfuric acid from a bottle fitted with a repipettor (i.e., automatic bottle dispenser). Fit the dispenser with a thin, semirigid tube to dispense directly and deep into the Babcock bottle while mixing contents. Set the bottle with dispenser on a tray to collect spills. Wear corrosive- and heat-resistant gloves when mixing the sulfuric acid with samples.

Procedure

1. Accurately pipette the milk sample (17.6 ml) into a Babcock test bottle (**Figure 5.12**).
2. Add reagent grade (1.82 specific gravity) sulfuric acid (17.5ml) to the bottle, allowing the acid to flow gently down the neck of the bottle as it is being slowly rotated. The acid digests proteins to liberate the fat.

3. Centrifuge the mixture for 5min and liquid fat will rise into the calibrated bottle neck. The centrifuge must be kept at 55-60 °C during centrifugation.
4. Add hot water to bring liquid fat up into the graduated neck of the Babcock bottle.
5. The direct percentage of fat by weight is read to the nearest 0.05% from the graduation mark of the bottle.

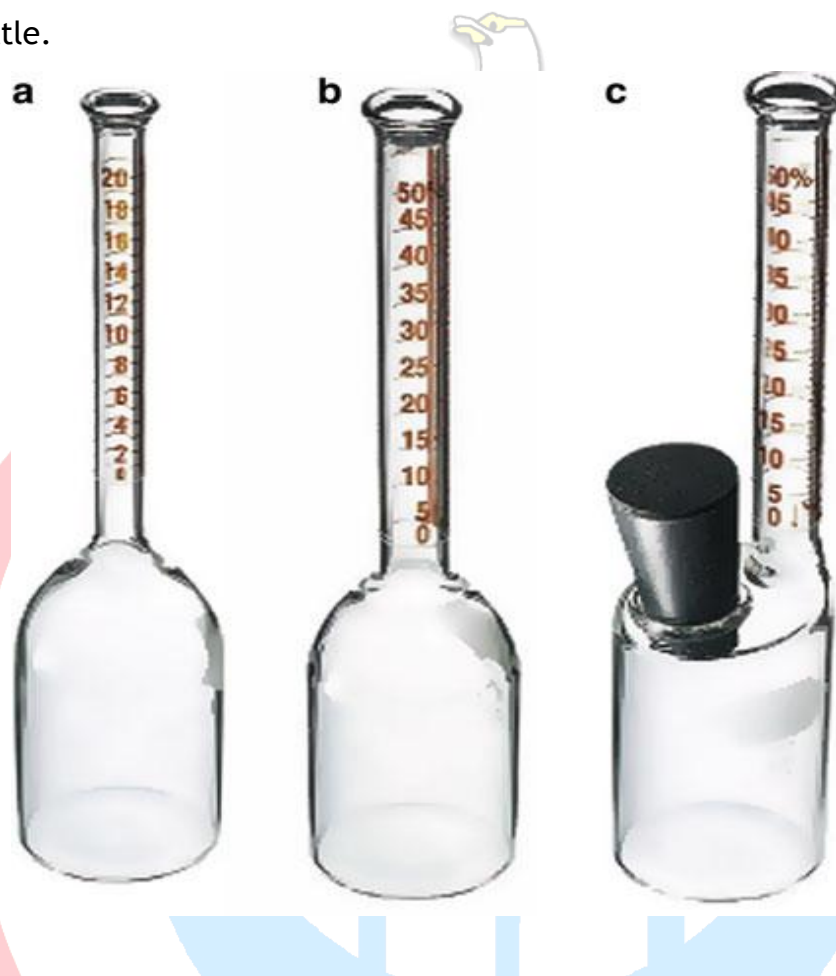


Fig. 5.12 Babcock milk test bottles for milk (a), cream (b), and cheese (Paley bottle) (c) testing.

Applications

The Babcock method, which is a common official method for the determination of fat in milk, takes about 45min and duplicate tests should agree within 0.1%. The Babcock method does not determine the phospholipids in the milk products. It is not applicable to products containing chocolate or added sugar without modification because of charring of chocolate and sugars by sulfuric acid. A modified Babcock method is used to determine essential oil in flavor extracts (AOAC Method 932.11) and fat in seafood (AOAC Method 964.12).

Gerber Method for Milk Fat

Principle

The principle of the Gerber method is similar to that of the Babcock method, but it uses sulfuric acid and amyl alcohol. The sulfuric acid **digests** proteins and carbohydrates, releases fat, and maintains the fat in a liquid state by generating heat.

Procedure

1. Transfer 10 ml of H_2SO_4 at 15-21 °C into a Gerber milk bottle.
2. Accurately measure milk sample (11 ml) into the Gerber bottle, using a Gerber pipette.
3. Add 1ml of isoamyl alcohol to the bottle.
4. Tighten the stopper and mix by shaking the bottle.
5. Centrifuge the bottle for 4 min.
6. Place the bottle in a water bath at 60-63 °C for 5min and then read the fat content from the graduations on the bottle neck.

Applications

The Gerber method is comparable to the Babcock method but is **simpler and faster** and has wider application to a variety of dairy products. The isoamyl alcohol generally prevents the charring of sugar found with the regular Babcock method.

Instrumental Methods

Infrared Method

The infrared (IR) method is based on **absorption of IR energy by fat** at a wavelength of 5.73 μm . The more the energy absorption at 5.73 μm , the higher is the fat content of the sample (15). Mid-IR spectroscopy is used in **Infrared Milk Analyzers** to determine milk fat content (AOAC Method 972.16). Near-infrared (NIR) spectroscopy has been used to measure the fat content of commodities such as meats, cereals, and oilseeds

Specific Gravity (Foss-Let Method)

Fat content by the Foss-Let method is determined as a function of the specific gravity of a sample solvent extract. A sample of known weight is extracted for 1.5 - 2 min in a vibration-reaction chamber with perchloroethylene. The extract is filtered, and using a thermostatically controlled device with digital readout, its specific gravity is determined. The reading can then be converted to oil or fat percentage using a conversion chart.

Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) can be used to measure lipids in food materials in a nondestructive way. It is one of the most popular methods for use in determining lipid-melting curves to measure solid fat content and with more affordable instruments is becoming more popular for measuring total fat content.

NMR is based on the observation that certain nuclei will absorb and re-emit **radio frequency (RF) energy** over a narrow band of frequencies when placed in a static magnetic field. The frequency at which the NMR effect occurs for a given nuclear isotope is dependent on the strength of the magnetic field of the magnet, and the phenomenon is caused by the interaction between the nuclear magnetic dipole of a nucleus and the magnetic field it experiences. (The latter is the reason that the word “nuclear” is included in the description of the phenomenon. NMR does not involve the emission of ionizing radiation.).

NMR analysis is a very **rapid and accurate** method, and while the principles of NMR are relatively complex, the use of NMR can be quite simple, especially due to the high degree of automation and computer control.

Protein Analysis

Proteins are polymers of amino acids, the majority of which are α -amino acids having the general formula $\text{NH}_2\text{CHR}\text{COOH}$, and may thus be distinguished from fats and carbohydrates in being the only macronutrient in foods to contain nitrogen. The presence of **nitrogen** in proteins is often used as the basis of the estimation of protein in foods.

Numerous methods have been developed to measure protein content. The basic principles of these methods include the determinations of nitrogen, peptide bonds, aromatic amino acids, dye-binding capacity, ultraviolet absorptivity of proteins, and light scattering properties. In addition to factors such as sensitivity, accuracy, precision, speed, and cost of analysis, what is actually being measured must be considered in the selection of an appropriate method for a particular application.

Importance of Analysis

Protein analysis is important for:

1. **Nutrition labeling**
2. **Pricing:** The cost of certain commodities is based on the protein content as measured by nitrogen content (e.g., cereal grains; milk for making certain dairy products, e.g., cheese).
3. **Functional property investigation:** Proteins in various types of food have unique food functional properties: for example, gliadin and glutenins in wheat flour for bread making, casein in milk for coagulation into cheese products, and egg albumen for foaming, and other functional properties such as water binding capacity and emulsifying characteristics.
4. **Biological activity determination:** Some proteins, including enzymes or enzyme inhibitors, are relevant to food science and nutrition: for instance, the proteolytic enzymes in the tenderization of meats, pectinases in the ripening of fruits, and trypsin inhibitors in legume seeds are proteins. To compare between samples, enzymes activity often is expressed in terms of specific activity, meaning units of enzyme activity per mg of protein.

Protein analysis is required when you want to know:

1. Total protein content
2. Content of a particular protein in a mixture
3. Protein content during isolation and purification of a protein
4. Non-protein nitrogen
5. Amino acid composition
6. Nutritive value of a protein

Content in Foods

Protein content in food varies widely. Foods of animal origin and legumes are excellent sources of proteins. The protein contents of selected food items are listed in **Table 5.5**.

Table 5.5 Protein content of selected foods

Food	Percent Protein (Wet Weight Basis)
White rice, raw	7.1
Wheat flour, whole-grain	13.7
Corn flour, whole-grain, yellow	6.9
Cornstarch	0.3
Milk, reduced fat, fluid, 2%	3.2
Cheese, cheddar	24.9
Ricotta cheese	11.26
Cheddar cheese	33.1
Yoghurt, plain, low fat	5.3
Apple, raw, with skin	0.3
Strawberries, raw	0.7
Potato, whole, flesh and skin	2.0
Beef, cured, dried beef	31.1
Chicken, broilers or fryers	23.1
Egg, raw, whole, fresh	12.6
Fish	20.4

From US Department of Agriculture, Agricultural Research Service (2009). USDA National Nutrient Database for Standard Reference. Home Page, <http://www.ars.usda.gov/ba/bhnrc/ndl>.

Methods of Analysis

Principles, general procedures, and applications are described below for various protein determination methods. Refer to the referenced methods for detailed instructions of the procedures. The Kjeldahl, Dumas (N combustion), and infrared spectroscopy methods cited are from the Official Methods of Analysis of AOAC International and are used commonly in nutrition labeling and quality control. The other methods described are used commonly in research laboratories working on proteins.

Kjeldahl Method

Principle

In the Kjeldahl procedure, proteins and other organic food components in a sample are digested with sulfuric acid in the presence of catalysts. The **total organic nitrogen** is converted to **ammonium sulfate**.

The digest is **neutralized** with alkali and distilled into a boric acid solution. The borate anions formed are titrated with standardized acid, which is converted to **nitrogen** in the sample. The result of the analysis represents the crude protein content of the food since nitrogen also comes from non-protein components (note that the Kjeldahl method also measures nitrogen in any ammonia and ammonium sulfate).

The basic **AOAC** Kjeldahl procedure is Method **955.04**. Semiautomation, automation, and modification for microgram nitrogen determination (micro Kjeldahl method) have been established by **AOAC** in Methods **976.06**, **976.05**, and **960.52**, respectively.

General Procedures and Reactions

1. **Sample Preparation** Solid foods are ground to pass a 20-mesh screen. Samples for analysis should be homogeneous. No other special preparations are required.
2. **Digestion** Place sample (accurately weighed) in a Kjeldahl flask. Add acid and catalyst; digest until clear to get complete breakdown of all organic matter. Nonvolatile ammonium sulfate is formed from the reaction of nitrogen and sulfuric acid.

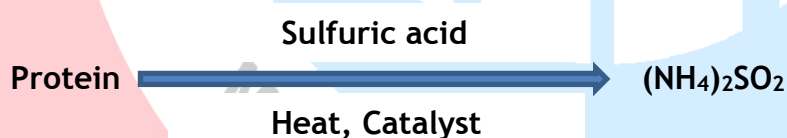


Fig. 5.13 Kjeldahl digestion unit

During digestion, protein nitrogen is released to form ammonium ions; sulfuric acid oxidizes organic matter and combines with ammonium formed; carbon and hydrogen elements are converted to carbon dioxide and water.

3. Neutralization and Distillation

The digest is diluted with water. Alkali-containing sodium thiosulfate is added to neutralize the sulfuric acid. The ammonia formed is distilled into a boric acid solution containing the indicators bromocresol green and methyl red (AOAC Method 991.20).

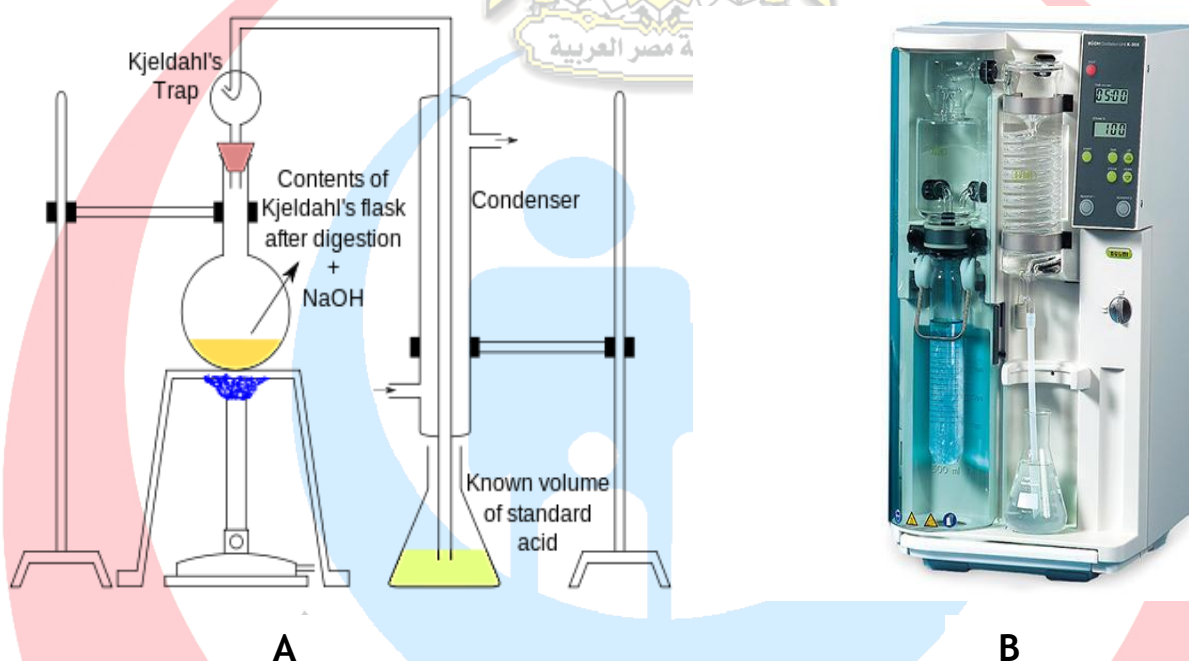


Fig. 5.14 Kjeldahl distillation unit, (A) Manual unit, (B) Automated unit

4. Titration Borate anion (proportional to the amount of nitrogen) is titrated with standardized HCl.



Personal protection

The wearing of protective glasses and acid resistant gloves is obligatory due to the risk of splashing of boiling sulfuric acid and breakage of tubes.

Calculations

Moles of HCl = moles of NH₃ = moles of N in the sample

A reagent blank should be run to subtract reagent nitrogen from the sample nitrogen.

$$\%N = \frac{NHCl \times \text{Corrected acid volume}}{\text{g of sample} \times 1000} \times 14 \times 100 \quad \text{Eq. 5.12}$$

Where:

NHCl = normality of HCl, in mol/1000 ml

Corrected acid vol. = (ml std. acid for sample) - (ml std. acid for blank)

14 = atomic weight of nitrogen

A factor is used to convert percent N to percent crude protein. Most proteins contain 16% N, so the conversion factor is 6.25 (100/16 = 6.25).

$$\% N / 0.16 = \% \text{ protein} \quad \text{Eq. 5.13}$$

or

$$\% N \times 6.25 = \% \text{ protein} \quad \text{Eq. 5.14}$$

Conversion factors for various foods are given in Table 5.6.

Table 5.6 Nitrogen to Protein Conversion Factors for Various Foods

	Percent N in Protein	Factor
Egg or meat	16.0	6.25
Milk	15.7	6.38
Wheat	18.76	5.33
Corn	17.70	5.65
Oat	18.66	5.36
Soybean	18.12	5.52
Rice	19.34	5.17

Formol titration

When formaldehyde (methanal) is added to a previously neutralized food, the formaldehyde reacts with the side chains of basic amino acid residues such as lysine. This results in the conversion of -NH₂ groups to -N=CH₂ groups with a consequent loss of basic properties and an increase in the acidity of the protein. The increase in acidity is then measured by titration with

standard sodium hydroxide using phenolphthalein as indicator and the increase in acidity is correlated with protein concentration from previous calibrations or a known conversion factor. The procedure may be used for estimating the protein content of milk given that:

$$\% \text{ protein} = T \times 0.17 \quad \text{Eq. 5.15}$$

Where

T = ml M NaOH required to neutralize the acidity produced in 1000 ml milk. It may also be used for estimating the non-fat milk solids present in ice cream.

The procedure is rapid and simple to perform but tends to underestimate the protein content, particularly in the case of milk protein.

Dumas (Nitrogen Combustion) Method

Principle

Samples are combusted at high temperatures (700-1000 °C) with a flow of pure oxygen. All carbon in the sample is converted to carbon dioxide during the flash combustion. Nitrogen-containing components produced include N_2 and nitrogen oxides.

The nitrogen oxides are reduced to nitrogen in a copper reduction column at a high temperature (600 °C). The total nitrogen (including inorganic fraction, i.e., including nitrate and nitrite) released is carried by pure helium and quantitated by **gas chromatography (GC)** using a **thermal conductivity detector (TCD)**. Ultra-high purity acetanilide and EDTA (ethylenediamine tetraacetate) may be used as the standards for the calibration of the nitrogen analyzer. The nitrogen determined is converted to protein content in the sample using a protein conversion factor.

Procedure

Samples (approximately 100-500 mg) are weighed into a tin capsule and introduced to a combustion reactor in automated equipment. The nitrogen released is measured by a built-in gas chromatograph. **Figure 5.15** shows the flow diagram of the components of a Dumas nitrogen analyzer.

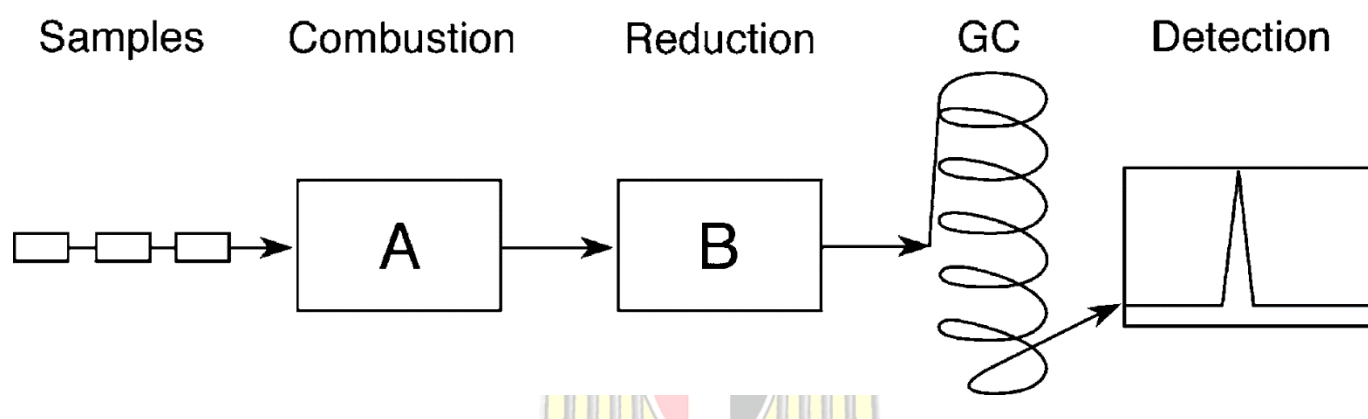


Fig. 5.15 General components of a Dumas nitrogen analyzer. A, the incinerator; B, copper reduction unit for converting nitrogen oxides to nitrogen; and GC, gas chromatography column.

Applications

The combustion method is an alternative to the Kjeldahl method and is suitable for all types of foods. **AOAC Method 992.15** and **Method 992.23** are for meat and cereal grains, respectively.

Advantages:

1. Requires no hazardous chemicals.
2. Can be accomplished in 3 min.
3. Recent automated instruments can analyze up to 150 samples without attention.

Disadvantages:

1. Expensive equipment is required.
2. Measures total organic nitrogen, not just protein nitrogen.

Infrared Spectroscopy

Principle

Infrared spectroscopy measures the **absorption of radiation** (near- or mid-infrared regions) by molecules in food or other substances. Different **functional groups** in a food absorb different frequencies of radiation. For **proteins and peptides**, various **mid-infrared** bands (6.47 μm) and **near-infrared** (NIR) bands (e.g., 3300-3500 nm; 2080-2220 nm; 1560-1670 nm) characteristic of the peptide bond can be used to estimate the protein content of a food. By irradiating a sample with a wavelength of infrared light specific for the constituent to be measured, it is possible to predict the concentration of that constituent by measuring the energy that is reflected or transmitted by the sample (which is inversely proportional to the energy absorbed).

Applications

Mid-infrared spectroscopy is used in Infrared Milk Analyzers to determine milk protein content, while near-infrared spectroscopy is applicable to a wide range of food products (e.g., grains; cereal, meat, and dairy products) (**AOAC Method 997.06**). Instruments are expensive and they must be calibrated properly. However, samples can be analyzed rapidly (30 s to 2 min) by analysts with minimal training.

Biuret Method

Principle

The Biuret method is based on the complexation of Cu^{+2} to functional groups in the protein's peptide bonds under alkaline conditions. The formation of a Cu^{+2} protein complex requires **two** peptide bonds and produces a **violet-purplish color**, which is measured by absorption spectroscopy at **540 nm**. Over a given concentration range, the measured absorption at 540 nm is linear with respect to the concentration of total protein. The color intensity (absorbance) is proportional to the protein content of the sample. This relationship allows a standard curve to be created that is used to calculate the concentration of an unknown sample.

Procedure

1. A 5-ml biuret reagent is mixed with a 1 ml portion of protein solution (1-10 mg protein/ml). The reagent includes copper sulfate, NaOH, and potassium sodium tartrate, which is used to stabilize the cupric ion in the alkaline solution.
2. After the reaction mix is allowed to stand at room temperature for 15 or 30 min, the absorbance is read at 540 nm against a reagent blank.
3. Filtration or centrifugation before reading absorbance is required if the reaction mixture is not clear.
4. A standard curve of concentration versus absorbance is constructed using bovine serum albumin (BSA).

Applications

The biuret method has been used to determine proteins in cereal, meat and soybean proteins. The biuret method also can be used to measure the protein content of isolated proteins.

Advantages:

1. Less expensive than the Kjeldahl method; rapid (can be completed in less than 30min); simplest method for analysis of proteins.
2. Very few substances other than proteins in foods interfere with the biuret reaction.
3. Does not detect nitrogen from non-peptide or non-protein sources.

Disadvantages:

1. Not very sensitive as compared to the Lowry method; requires at least 2-4 mg protein for assay.
2. Absorbance could be contributed from bile pigments if present.
3. High concentration of ammonium salts interfere with the reaction.
4. Color varies with different proteins; gelatin gives a pinkish-purple color.
5. Opalescence could occur in the final solution if high levels of lipid or carbohydrate are present.
6. Not an absolute method: color must be standardized against known protein (e.g., BSA) or against the Kjeldahl nitrogen method.

Dye-Binding Methods**A. Anionic Dye-Binding Method**

Principle. The protein-containing sample is mixed with a known excess amount of **anionic dye** in a buffered solution. Proteins bind the dye to form an insoluble complex. The unbound soluble dye is measured after equilibration of the reaction and the removal of insoluble complex by centrifugation or filtration.

Protein + excess dye → **Protein -dye insoluble complex + unbound soluble dye**

The anionic sulfonic acid dye, including acid orange 12, orange G, and Amido Black 10 B, binds cationic groups of the basic amino acid residues (imidazole of histidine, guanidine of arginine, and ϵ -amino group of lysine) and the free amino terminal group of the protein. The amount of the **unbound dye** is inversely related to the **protein content** of the sample.

Procedure

1. The sample is finely ground (60 mesh or smaller sizes) and added to an excess dye solution with known concentration.
2. The content is vigorously shaken to equilibrate the dye binding reactions and filtered or centrifuged to remove insoluble substances.
3. Absorbance of the unbound dye solution in the filtrate or supernatant is measured and dye concentration is estimated from a dye standard curve.
4. A straight calibration curve can be obtained by plotting the unbound dye concentration against total nitrogen (as determined by Kjeldhal method) of a given food covering a wide range of protein content.
5. Protein content of the unknown sample of the same food type can be estimated from the calibration curve.

Applications

Anionic dye binding has been used to estimate proteins in milk, wheat flour, soy products, and meats. The AOAC approved methods include two dye-binding methods [Method 967.12 using Acid Orange 12 and Method 975.17 using Amido Black (10 B) for analyzing proteins in milk].

Advantages:

1. Rapid (15 min or less), inexpensive, and relatively accurate for analyzing protein content in food commodities.
2. May be used to estimate the changes in available lysine content of cereal products during processing since the dye does not bind altered, unavailable lysine. Since lysine is the limiting amino acid in cereal products, the available lysine content represents protein nutritive value of the cereal products.
3. No corrosive reagents.
4. Does not measure non-protein nitrogen.
5. More precise than the Kjeldahl method.

Disadvantages:

1. Not sensitive; milligram quantities of protein are required.
2. Proteins differ in basic amino acid content and so differ in dye-binding capacity. Therefore, a calibration curve for a given food commodity is required.
3. Not suitable for hydrolyzed proteins due to binding to N-terminal amino acids.
4. Some non-protein components bind dye (i.e., starch) or protein (calcium or phosphate) and cause errors in results. The problem with calcium and heavy metal ions can be eliminated using properly buffered reagent that contains oxalic acid.

B. Bradford Dye-Binding Method

Principle. When Coomassie Brilliant Blue G-250 binds to protein, the dye changes color from reddish to bluish and the absorption maximum of the dye is shifted from 465 to 595 nm. The change in the absorbance at 595 nm is proportional to the protein concentration of the sample.

Procedure

1. Coomassie Brilliant Blue G-250 is dissolved in 95% ethanol and acidified with 85% phosphoric acid.
2. Samples containing proteins (1-100 µg/ml) and standard BSA solutions are mixed with the Bradford reagent.

3. Absorbance at 595 nm is read against a reagent blank.
4. Protein concentration in the sample is estimated from the BSA standard curve.

Applications

The Bradford method has been used successfully to determine protein content in potato tubers. This procedure has been improved to measure microgram quantities of proteins.

Advantages:

1. Rapid; reaction can be completed in 2 min
2. Reproducible
3. Sensitive; several fold more sensitive than the Lowry method
4. Measures protein or peptides with molecular mass approximately equal to or greater than 4000Da

Disadvantages:

1. The protein-dye complex can bind to quartz cuvettes. The analyst must use glass or plastic cuvettes.
2. Color varies with different types of proteins. The standard protein must be selected carefully.

Ultraviolet 280nm Absorption Method

Principle

Proteins show strong absorption in the region at ultraviolet (UV) 280nm, primarily due to tryptophan and tyrosine residues in the proteins. Because the content of tryptophan and tyrosine in proteins from each food source is fairly constant, the absorbance at 280 nm could be used to estimate the concentration of proteins, using **Beer's law**.

Procedure

1. Proteins are solubilized in buffer or alkali.
2. Absorbance of protein solution is read at 280 nm against a reagent blank.
3. Protein concentration is calculated according to the equation

$$A = alc$$

Eq. 5.16

Where:

A = absorbance

a = absorptivity

l = cell or cuvette path length

c = concentration

Applications

The UV 280-nm method has been used to determine the protein contents of milk and meat products.

Advantages:

1. Rapid and relatively sensitive; At 280 nm, 100 μg or more protein is required; several times more sensitive than the biuret method.
2. No interference from ammonium sulfate and other buffer salts.
3. Nondestructive; samples can be used for other analyses after protein determination.

Disadvantages:

1. Nucleic acids also absorb at 280nm.
2. Aromatic amino acid contents in the proteins from various food sources differ considerably.
3. The solution must be clear and colorless. Turbidity due to particulates in the solution will increase absorbance falsely.
4. A relatively pure system is required to use this method, so it has not been used widely in food systems.



Carbohydrate Analysis

Carbohydrates are among the most important ingredients in foods and raw materials. They not only act as energy sources but also impart crucial textural properties and have physiologically beneficial effects.

Carbohydrates are generally classified into sugar alcohols (e.g. sorbitol), monosaccharides (e.g. glucose), oligosaccharides (e.g. raffinose), and polysaccharides (e.g. starch and nonstarch polysaccharides).

It should be noted that according to the nutrition labeling regulations of the US Food and Drug Administration, the “**total carbohydrate**” content of a food (**Table 5.7**), which is declared in relation to a serving, which is defined as the amount of food customarily consumed per eating occasion by person 4-years of age or older, must be calculated by subtraction of the sums of the weights of crude protein, total fat, moisture, and ash in a serving from the total weight of the food in a serving (i.e., carbohydrate is determined by difference). The grams of dietary fiber in a serving also must be stated on the label. The content of “other carbohydrate” (formerly called “**complex carbohydrate**”) is obtained by calculating the difference between the amount of “total carbohydrate” and the sum of the amounts of dietary fiber and sugars. For labeling purposes, sugars are defined as the sum of all free **monosaccharides** (viz., D-glucose and -fructose) and **disaccharides** [viz., sucrose, lactose, and maltose (if a maltodextrin or glucose/corn syrup has been added)].

Sample Preparation

Sample preparation is related to the specific raw material, ingredient, or food product being analyzed and the specific carbohydrate being determined, because carbohydrates have such a wide range of solubilities. However, some generalities can be presented (**Figure 5.16**).

For most foods, the first step is drying, which also can be used to determine moisture content. For other than beverages, drying is done by placing a weighed amount of material in a vacuum oven and drying to constant weight at 55 °C and 1mm Hg pressure. Then, the material is ground to a fine powder, and lipids are extracted using 19:1 vol/vol chloroform-methanol in a Soxhlet extractor. Prior extraction of lipids makes extraction of carbohydrates easier and more complete.

Water-soluble polysaccharides can be extracted using hot water and separated from insoluble material by centrifugation or filtration. Part of the insoluble polysaccharides could be obtained using mild base extraction (KOH or NaOH with the concentration less than 0.5 M). Starch could be removed by an enzymatic treatment with α -amylase and/or amyloglucosidase. Protein can be removed by physical and enzymatic or chemical methods (protease or isoelectric precipitation).

Table 5.7 Total Carbohydrate Contents of Selected Foods

Food	Approximate Percent Carbohydrate (Wet Weight Basis)
Cereals, bread, and pasta Corn flakes	80.4
Macaroni, dry	74.7
Bread, white, commercially prepared	50.6
Ice cream, chocolate	28.2
Milk, reduced fat, fluid, 2%	4.7
Whole milk, chocolate, commercial	10.3
Apple sauce, canned, sweetened	19.9
Grapes, raw	17.2
Apple, raw, with skin	13.8
Potatoes, raw, with skin	12.4
Orange juice, raw	10.4
Carrots, raw	9.6
Broccoli, raw	6.6
Fish fillets, battered or breaded, fried	17.0
Beef	4.0
Chicken, broilers or fryers	0.0
Honey	82.4

From US Department of Agriculture, Agricultural Research Service (2009). USDA National Nutrient Database for Standard Reference. Home Page, [http:// www.ars.usda.gov/ba/bhnrc/ndl](http://www.ars.usda.gov/ba/bhnrc/ndl)

Methods of Analysis

By difference. This involves obtaining the available carbohydrate content by calculation having estimated all the other fractions by proximate analysis

$$\% \text{ Available carbohydrates} = 100 - [\% \text{ Moisture} + \% \text{ Ash} + \% \text{ Fat} + \% \text{ Protein} + \% \text{ Fibre}] \quad \text{Eq. 5.17}$$

The procedure has the advantage of not having to perform a separate determination for these carbohydrates and is used as part of the calculation of the energy value of a food. It has the disadvantage of a potentially high error of estimation due to the possible errors incurred in each of the proximate determinations.

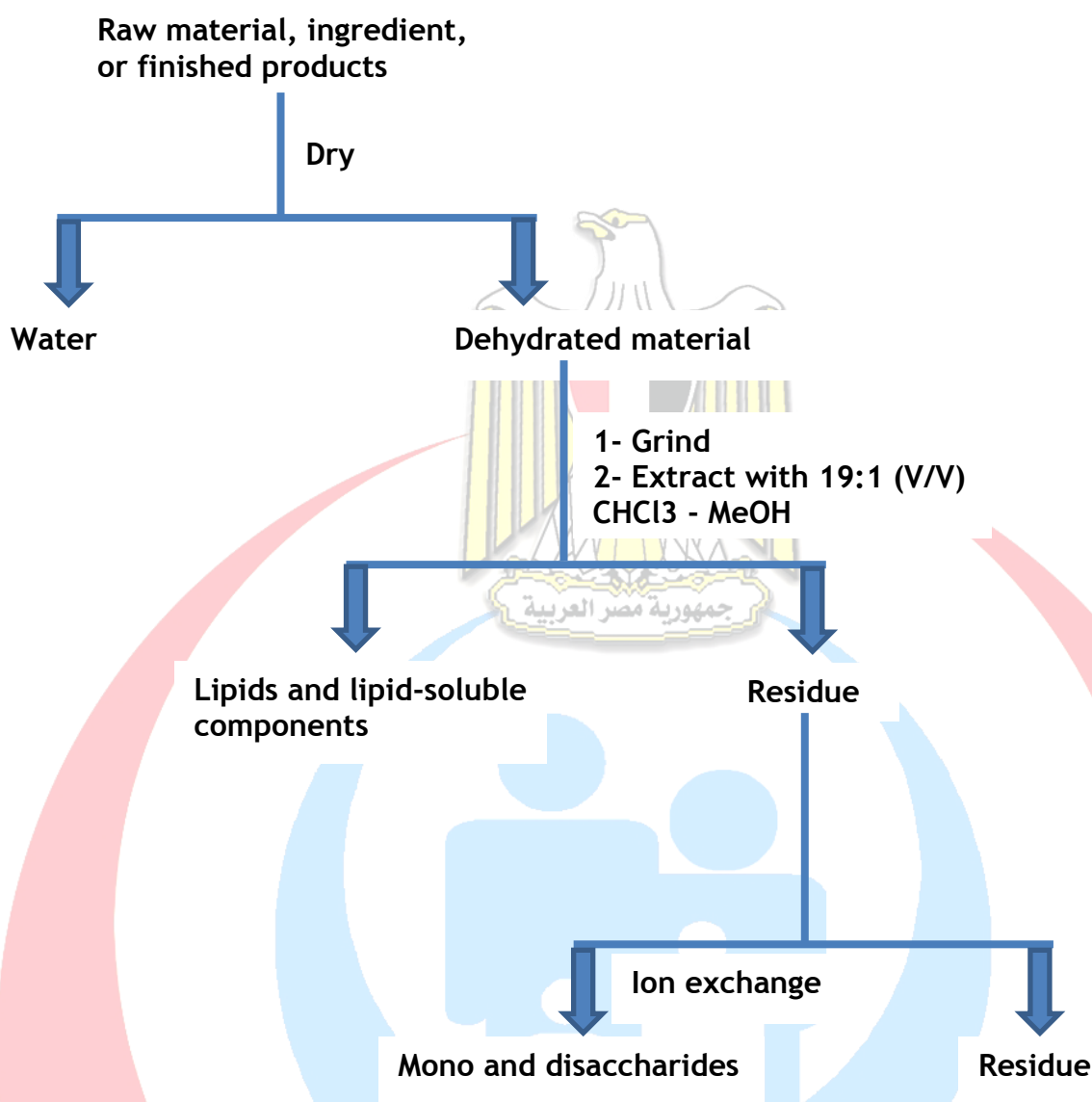


Fig. 5.16 Flow diagram for sample preparation and extraction of mono- and disaccharides.

Physical methods for carbohydrate analysis:

Polarimetry. This procedure, which makes use of instruments known as polarimeters, is based on the principle that sugars are optically active, i.e. they will rotate the direction of a beam of plane polarised light (light of one wavelength moving in one plane), the amount of rotation being dependent on the concentration of sugars in solution as in the relationship:

$$[\alpha]_{\lambda}^T = \alpha / L.C$$

In this equation, widely used in food analysis, L is the path length in decimeters and c is the concentration in g/mL, for a pure compound at a temperature T (given in degrees Celsius) and wavelength λ (in nanometers). The specific rotation of different mono- and oligosaccharides has been listed in previous reports. A **calibration curve** of specific rotation versus concentration is prepared using a series of solutions with known concentration, or the value of specific

rotation can be taken from the literature. The concentration of carbohydrate in an unknown sample is then determined by measuring its angle of rotation and comparing it with the calibration curve.

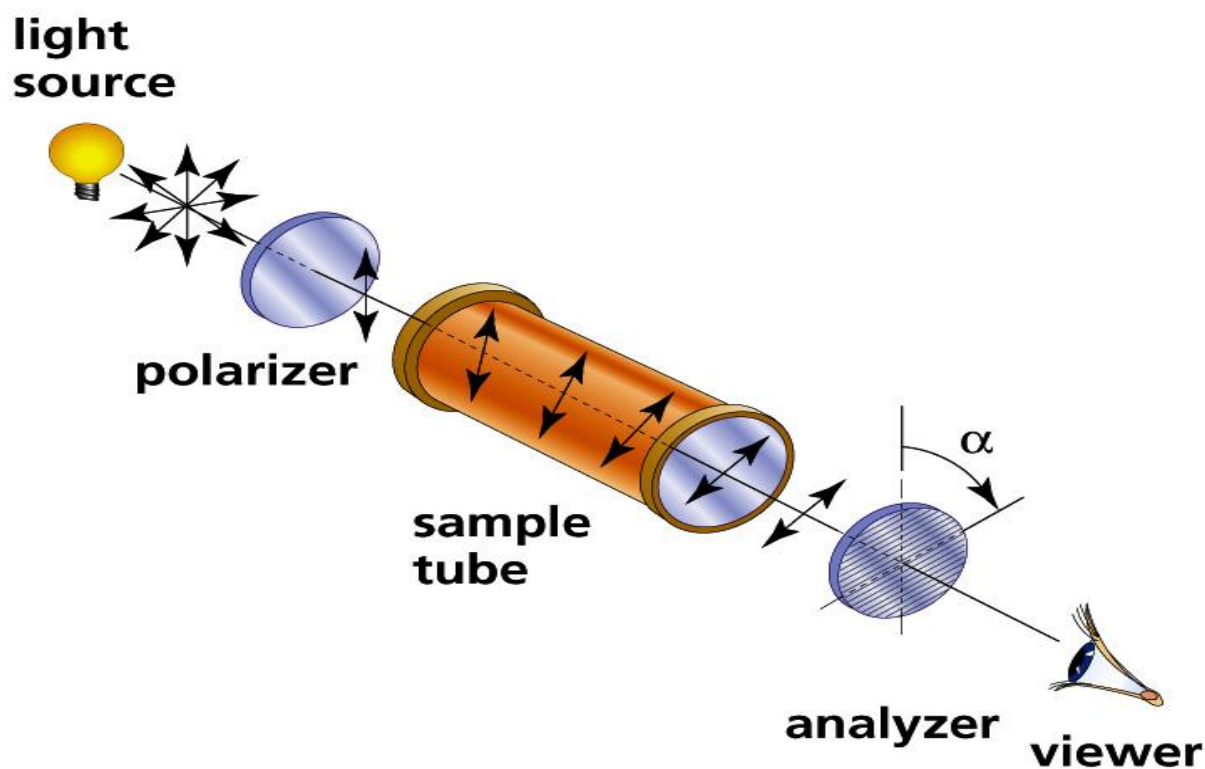


Fig. 5.17 Description of *Polarimetric procedure*



Fig. 5.18 Manual Polarimeter (A) and digital Polarimeter (B).

Refractometry.

The method is used to determine the sugar content of syrups, fruit juices, ferments, vegetable juices and milk-based drinks, and to estimate the total concentration of **monosaccharides** and **disaccharides** in any solution.

What is the refractive index?

The **refractive index (RI)** of a substance is the ratio between the velocity of light in a vacuum and its velocity in the Substance.

As light passes from one medium, such as air, into another medium such as an aqueous solution (**Figure 5.19**), the light rays are refracted (bent) and the refractive index, μ , of the solution is given by the relationship:

$$\mu = \sin i / \sin r$$

Eq. 5.18

Where i is the angle of incidence (the angle between the incident ray and the vertical) and r is the angle of refraction (the angle between the emergent ray and the vertical).

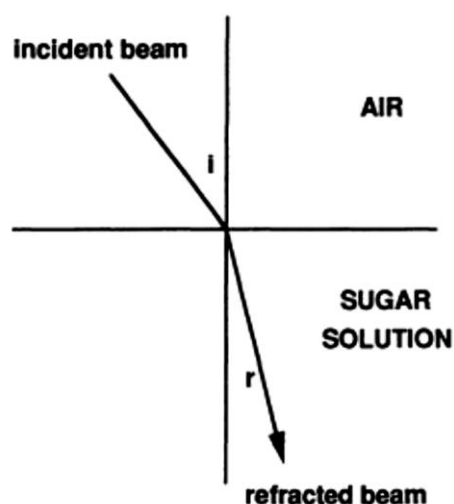


Fig. 5.19 Principle of refractometry showing refraction of a beam of light travelling from air to a sugar solution.

The refractive index of a solution is dependent on the concentration of materials in solution and refractometry is thus a rapid and convenient way of estimating food components such as the sugar contents of jams, etc.

Refractometer is an optical device that can be used to measure the refractive index of a fluid substances and, by converting the refractive index, one can get the specific gravity, sugar content (**Brix**).

If you start with a glass of clear water, you will notice that the water and glass bend the light passing through it in a certain way. The bending of the light by the water is called refraction. Light bends to different degrees as it passes through different substances. If you add sugar to your glass of water, the light will bend more. The refractometer takes advantage of this effect to measure the amount of bending (refraction) which indicates the amount of sugar in the sample. Most refractometers use a prism and a light source to illuminate the sample. On inexpensive refractometers, you hold the instrument up to a natural light source. More expensive models have internal light sources.

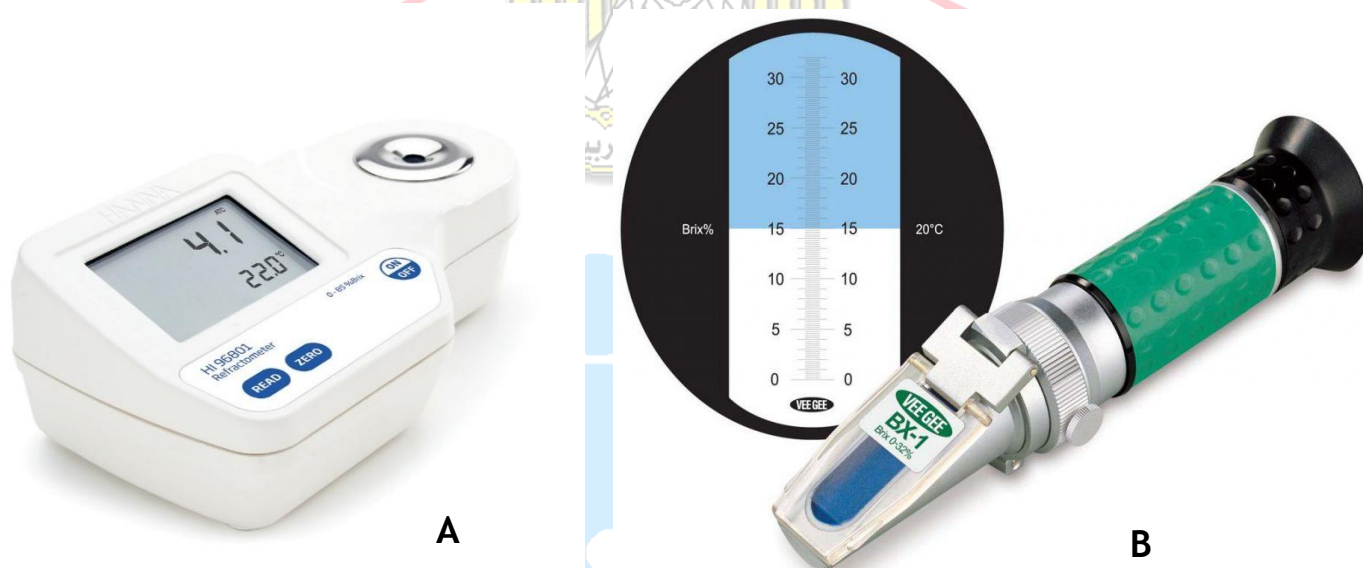


Fig. 5.20 Bench-top (A) and portable (B) refractometer instrument

In practice, instruments known as **saccharimeters**, which are calibrated directly in percentage of the sugar being estimated, are used. This allows a more rapid estimation of the sugar than would be the case using general refractometers which would require **pre-calibration**.

Calibration process of refractometer

Before you use a refractometer, it needs to be calibrated. Most refractometers are calibrated by using a sample of distilled water.

If you want an accurate reading, you should also calibrate the refractometer using reference sample that has a known sugar ratio.

Specific gravity (hydrometers)

Specific gravity is the **ratio** of density of a substance and the density of a reference sample. Water is usually selected as reference for liquids. This property is commonly used in the industry to determine the concentration of sugar solutions (syrops, juices, honey, etc.) (**AOAC 932.14**). In a food matrix, due to its complex carbohydrate composition, this method cannot provide accurate data, but for single pure sugar solutions, such as those of sucrose, it is rapid and accurate. Hydrometers can be used to estimate the specific gravity value of a solution. The data obtained (°Brix or °Baume) is converted to concentrations by use of tables constructed for the substance in pure solution.

Total sugar Analysis

Phenol-Sulfuric Acid Method

Principle. Phenol-sulfuric acid assay is a very sensitive classical method for the determination of total sugar content of carbohydrates including simple sugars, oligosaccharides, polysaccharides, and their derivatives (such as the methyl ethers with free or potentially free reducing groups). By heating in presence of acids, carbohydrates undergo a series of dehydration reactions (**Figure 5.21**) followed by the formation of furan derivatives (**Figure 5.22**). The dark-colored compounds are then formed through the condensation of furan derivatives with themselves or with phenolic compounds. These complexes could be monitored by spectrophotometer. The UV-VI absorbance is proportional to the sugar concentration. The maximum absorbance is observed at **490 nm** for hexoses and **480 nm** for pentoses and uronic acids.

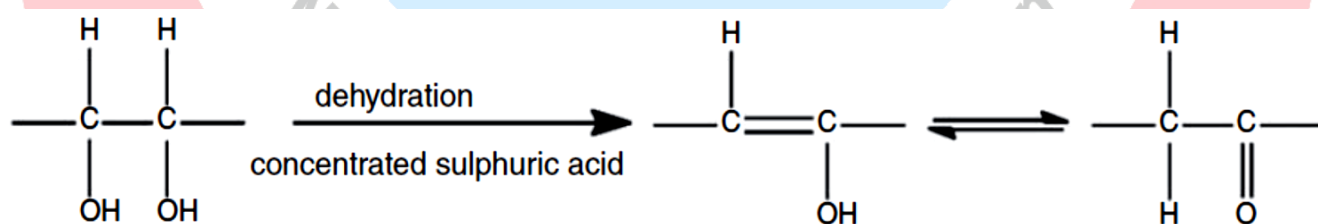


Fig. 5.21 Dehydration reaction

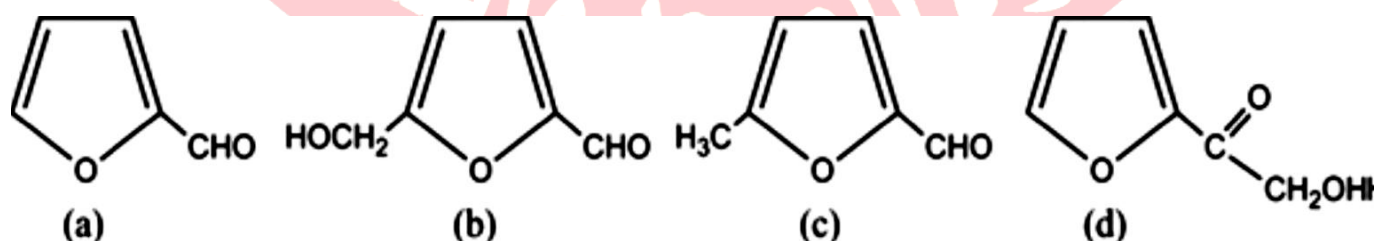


Fig. 5.22 Furan derivatives from (a) pentose and hexuronic acid (b) hexoses (c) 6-deoxyhexoses (d) keto-hexoses, respectively

Chemicals

Reagent	Hazards
D-Glucose ($C_6H_{12}O_6$)	
Phenol (C_6H_6O)	Toxic
Sulfuric acid (H_2SO_4)	Corrosive

Hazards, Cautions, and Waste Disposal

Use concentrated H_2SO_4 and the 80% phenol solution with caution. Wear gloves and safety glasses at all times, and use good lab technique. The concentrated H_2SO_4 is very corrosive (e.g., to clothes, shoes, skin). The phenol is toxic and must be discarded as hazardous waste.

Outline of Procedure

1. A clear, aqueous solution of carbohydrate(s) is transferred using a pipette into a small tube. A blank of water also is prepared.
2. An aqueous solution of phenol is added, and the contents are mixed.
3. Concentrated sulfuric acid is added rapidly to the tube so that the stream produces good mixing. The tube is agitated. (Adding the sulfuric acid to the water produces considerable heat.). A yellow-orange color results.
4. Absorbance is measured at 490 nm.
5. The average absorbance of the blanks is subtracted, and the amount of sugar is determined by reference to a standard curve using graduated concentrations of the standard glucose solution (figure 5.23).

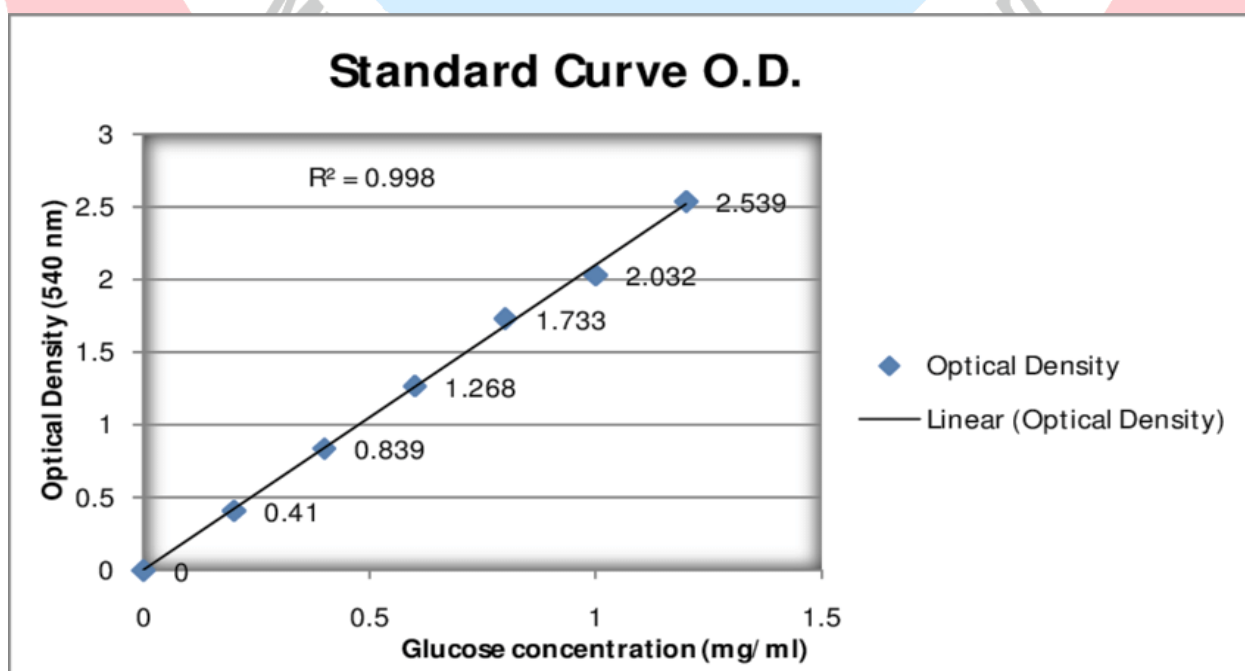


Fig. 5.23 Glucose standard curve for reducing sugar estimation.

This method is simple, rapid, sensitive, accurate, specific for carbohydrates, and widely applied. The reagents are inexpensive, readily available, and stable. Virtually all classes of sugars, including sugar derivatives and oligo- and polysaccharides, can be determined with the phenol-sulfuric acid method. The phenol-sulfuric acid procedure is often used as a qualitative test for the presence of carbohydrate.

Anthrone-Sulfuric Acid Method

Anthrone-sulfuric acid assay is also one of the most commonly used methods. This method has been used to measure soluble sugars in samples of different vegetal tissues of apple trees or maize plants.

Principles. The principle of this method is similar to the phenol-sulfuric method: with the presence of strong acid, the dehydration of the carbohydrate compounds leads to formation of furan derivatives, followed by reaction with the anthrone reagent to yield a **blue-green color**. The solution is then allowed to cool and measured at **620 nm** by spectrophotometer. Due to the presence of concentrated sulfuric acid, the non-reducing sugar could be converted to reducing sugar, therefore this method can be used to measure both reducing and non-reducing sugar contents (**total sugar contents**).

Operation procedure

Anthrone (0.2% w/v) in concentrated sulfuric acid is added to an aliquot of a measured sample. The mixture is kept in a temperature-controlled environment for sufficient time to allow a blue-green colored complex to develop (for example, at 15 °C for 30-45 min). The absorbance is determined at 620 nm by spectrophotometer. As same with other methods, the construction of **standard curve** (glucose is frequently used as standard) is required.

Measurement of total reducing sugar

DNS Method

Principle. 3,5 Dinitrosalicylic acid is an aromatic compound that reacts with reducing sugars (eg. Glucose, lactose..) to form 3-amino-5-nitrosalicylic acid, which absorbs light strongly at **540 nm** (in case of glucose). This method tests for the presence of **free carbonyl group** (C=O), the so called **reducing sugar**. This involves the oxidation of the aldehyde functional group present in, for example, glucose and the ketone functional group in fructose. Simultaneously, 3,5 Dinitrosalicylic acid is reduced to 3-amino-5-nitrosalicylic acid under alkaline conditions (**Figure 5.24**).

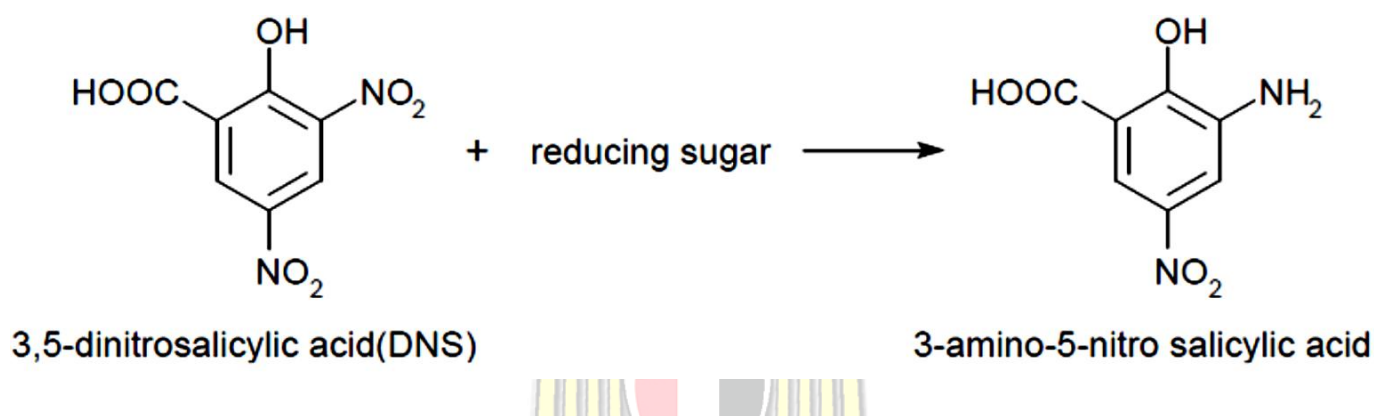


Fig. 5.24 The reaction between 3,5 Dinitrosalicylic acid and reduced sugars.

Somogyi-Nelson method

Principle. The Somogyi-Nelson method has been widely used for reducing sugar determinations. These sugars can reduce the copper from cupric $\text{Cu}(\text{OH})_2$ to cuprous state (Cu_2O) when heated with alkaline copper tartrate. The cuprous oxide could reduce arsenomolybdic acid (ammonium molybdate $[(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}]$ and sodium arsenate (Na_2HAsO_7) in sulfuric acid) to form **molybdenum blue**, which has an absorbance at **520 nm**. The absorbance is proportional to the reducing sugar contents in the sample.



A calibration curve should be established for quantification.

Operation procedure

CuSO_4 and an alkaline buffer are added into a solution of reducing sugars; this is followed by heating the solution in a boiling water bath for 20 min. An arsenomolybdate complex, prepared by reacting ammoniummolybdate $[(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}]$ and sodium arsenate (Na_2HAsO_7) in sulfuric acid is added. After mixing, absorbance is measured at 520 nm. A calibration curve should be established (usually with glucose as standard). After subtracting the absorbance of the reagent blank (water solution, without reducing sugar), the amount of total reducing sugar is calculated and reported, usually as glucose equivalent.

Other methods for reducing sugar analysis

Some methods that are frequently used to identify the presence of reducing sugars are listed below:

Fehling's test. Two types of solution are used in this test: **Fehling A** (a blue aqueous solution of copper (II) sulfate) and **Fehling B** (a clear solution of aqueous potassium sodium tartrate in strong alkali), which are stored separately before use. Equal amount of Fehling A and B solution are mixed to obtain the final Fehling's solution before measurement. The sample containing

reducing sugars will mixed with Fehling's solution; the **reducing sugars** will reduce copper (II) to copper (I) with a brick **red color** (Cu_2O) formed in the reactant solution. Fehling's test can distinguish aldehydes from ketones.

Benedict's test. Benedict's agent contains 173 g/L sodium citrate, 100 g/L sodium carbonate, and 17.3 g/L cupric sulfate pentahydrate. The reaction principle is similar to Fehling's test: copper (II) was reduced to copper (I) by reducing sugars. However, the color of the precipitate obtained (Cu_2O) is different depending on the content of the reducing sugars; for example, a greenish precipitate indicates about 0.5% concentration; yellow precipitate estimates 1% concentration; orange estimates 1.5% and red estimates 2% or higher concentration. Benedict's test can therefore be used to provide a semi-quantitative test for reducing sugars.

Chloramine-T method. In this procedure, an excess of Chloramine-T is added to a known volume of sugar solution and the solution is left in the dark for a period of about 1 h. In this time, reducing sugars are **oxidized** by the Chloramine-T, the latter being simultaneously reduced. At the end of this time, the remaining Chloramine-T is estimated by the addition of potassium iodide, which is oxidized by the remaining Chloramine-T to iodine. The iodine is estimated by titration with standard sodium thiosulphate solution using starch as indicator. This method is quick, simple to conduct and has found acceptance for estimating the lactose in milk.

Anion-exchange chromatography

With the development of advanced techniques, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has been widely used for monosaccharide and oligosaccharide analysis. At high pH (above 10), carbohydrate hydroxyl groups ionize and their separation is based on their differing affinity for the oppositely charged stationary phase and the mobile phase. It gives high resolution of all common monosaccharides and oligosaccharides. This method has the advantage of not requiring any derivatization before injection. However, the electrochemical response can be reduced by the presence of peptides and proteins. Student can find more detailed information on anion-exchange chromatographic analysis of in **chapter 8** of this book.

Gas liquid chromatography

Gas chromatography (GC) is still a valuable tool in the determination of monosaccharides and is widely used for carbohydrate analysis. Methods involving GC are **rapid and sensitive**. Excellent resolution and robustness are typically associated with GC applications owing to the large number of theoretical chromatographic plates and the inherent purity of the final derived sample dissolved in organic solvent. However, reduction and acetylation process are required

to convert carbohydrate into volatile compounds, which is complex and time consuming. The detailed principles and operating procedures of GC methods will be covered in **chapter 20** of this book.

Analysis of Dietary Fiber

Definition

Dietary fiber, an important food ingredient (either naturally occurring or as a food additive), presents numerous physiological properties such as, lowering cholesterol and glycaemic levels, trapping substances that can be dangerous for the human organism (mutagenic and carcinogenic agents), stimulating the proliferation of the intestinal flora, alleviating constipation.

Dietary fiber is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fiber promotes beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation.

Dietary fiber means **carbohydrate polymers with ten or more monomeric units**, which are not hydrolyzed by the endogenous enzymes in the small intestine of humans.

Enzymatic/gravimetric methods

The enzymatic/gravimetric methods mainly include: (i) enzyme hydrolysis (α -amylase, amyloglucosidase, and protease) to remove digestible material; (ii) an ethanol precipitation step to precipitate high molecular dietary fiber; (iii) ash and protein determination. The quantity of protein and ash is subtracted from the dry residue weight to obtain a total dietary fiber value. This method has been modified to permit the determination of total soluble and insoluble dietary fiber, whereas the soluble dietary fibers could be divided into soluble dietary fiber precipitate (SDFP) and alcohol soluble dietary fiber (SDFS). The flow chart for this method is given in **Figure 5.25**.

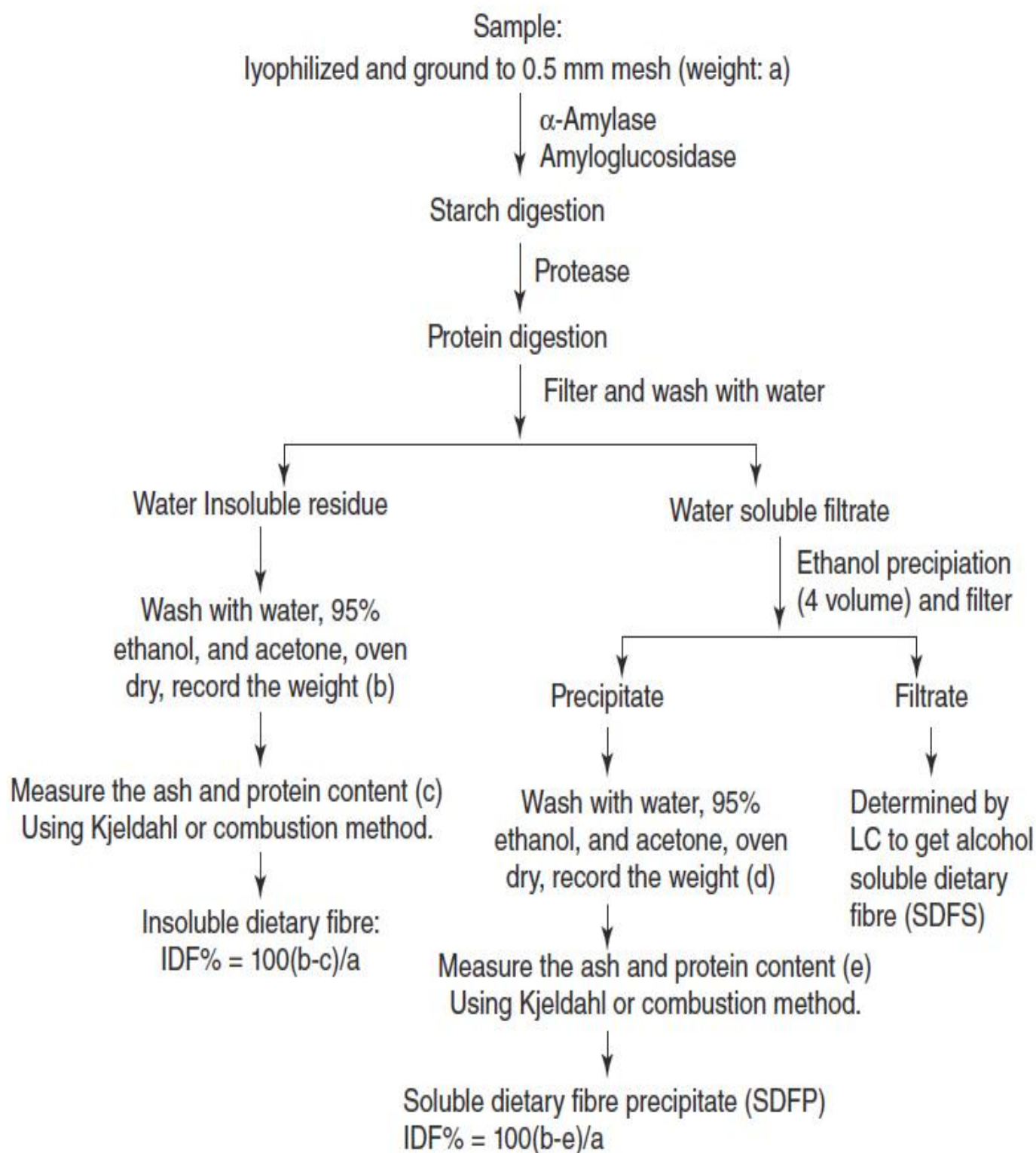


Fig. 5.25 Flow chart of soluble, insoluble and total dietary fiber determination by an enzymatic/gravimetric method.

Vitamin Analysis

Vitamins are defined as relatively low-molecular weight compounds which humans, and for that matter, any living organism that depends on organic matter as a source of nutrients, require small quantities for normal metabolism. With few exceptions, humans cannot synthesize most vitamins and therefore need to obtain them from food and supplements. Insufficient levels of vitamins result in deficiency diseases [e.g., scurvy and pellagra, which are due to the lack of ascorbic acid (vitamin C) and niacin, respectively].

Importance of Analysis

Vitamin analysis of food and other biological samples has played a critical role in determining animal and human nutritional requirements. Furthermore, accurate food composition information is required to determine dietary intakes to assess diet adequacy and improve human nutrition worldwide. From the consumer and industry points of view, reliable assay methods are required to ensure accuracy of food labeling.

Vitamin Units

When vitamins are expressed in units of mg or μg per tablet or food serving, it is very easy to grasp how much is present. Vitamins can also be expressed as international units (IU), United States Pharmacopeia (USP) units, and % Daily Value (DV). The IU is a unit of measurement for the amount of a substance, based on measured biological activity or effect. It is used for vitamins, hormones, vaccines, and similar biologically active substances. The precise definition of 1 IU differs from substance to substance, but has been established by international agreement for each substance. There is no equivalence among different substances; that is, 1 IU or USP unit of vitamin E does not contain the same number of micrograms as 1 IU or USP unit of vitamin A. For example, 1 IU of vitamin C is the biological equivalent of 50 μg L-ascorbic acid.

Vitamins are classified based on their solubility into:

Fat-soluble:

A, D, E, K

Water-soluble:

B complex, C

Methods of Analysis

Vitamin assays can be classified as follows:

1. **Bioassays** involving humans and animals.
2. **Microbiological assays** making use of protozoan organisms, bacteria, and yeast.
3. **Physicochemical assays** that include spectrophotometric, fluorometric, chromatographic, enzymatic, immunological, and radiometric methods.

Extraction Methods

With the exception of some biological feeding studies, vitamin assays in most instances involve the extraction of a vitamin from its biological matrix prior to analysis. This generally includes one or several of the following treatments: **heat, acid, alkali, solvents, and enzymes.**

In general, extraction procedures are specific for each vitamin and designed to stabilize the vitamin. In some instances, some procedures are applicable to the combined extraction of more than one vitamin, for example, for thiamin and riboflavin as well as some of the fat-soluble vitamins. Typical extraction procedures are as follows:

- **Ascorbic acid:** Cold extraction with metaphosphoric acid/acetic acid.
- **Vitamin B1 and B2:** Boiling or autoclaving in acid plus enzyme treatment.
- **Niacin:** Autoclaving in acid (nongrain products) or alkali (grain products).
- **Vitamins A, E, or D:** Organic solvent extraction, saponification, and re-extraction with organic solvents. For unstable vitamins such as these, antioxidants are routinely added to inhibit oxidation.

Analysis of fat-soluble vitamins may require saponification, generally either over-night at room temperature or by refluxing at 70°C. In the latter case, an air-cooled reflux vessel provides excellent control of conditions conducive to oxidation.

Methods available for vitamin assay include the following:

-**Microbiological.** Many of the B complex vitamins, such as thiamine and riboflavin, may be estimated by measuring the **growth of micro-organisms** having a specific requirement for the particular vitamin being assayed.

The procedure involves the preparation of a calibration curve from a set of standards of known vitamin concentrations and estimating the vitamin content of the food extract by comparison

against these standards. The growth of the micro-organisms may be measured on the basis of techniques such as carbon dioxide production or turbidity measurements.

Chemical Methods of Analysis

Vitamin C Determination by Indophenol Method

The vitamin (L-ascorbic acid and L-dehydroascorbic acid) is very susceptible to oxidative deterioration, which is enhanced by high pH and the presence of ferric and cupric ions. For these reasons, the entire analytical procedure needs to be performed at low pH and, if necessary, in the presence of a chelating agent.

The official method of analysis for Vitamin C determination of juices is the 2, 6-dichloroindophenol titrimetric method (AOAC Method 967.21). While this method is not official for other types of food products, it is sometime used as a rapid, quality control test for a variety of food products, rather than the more time-consuming microfluorometric method (AOAC Method 984.26).

Principle of Method

Ascorbic acid reduces the indicator dye to a colorless solution. At the endpoint of titrating an ascorbic acid containing sample with dye, excess unreduced dye is a rose-pink color in the acid solution. The titer of the dye can be determined using a standard ascorbic acid solution. Food samples in solution then can be titrated with the dye, and the volume for the titration used to calculate the ascorbic acid content.

Chemicals

Reagent

Acetic acid (CH_3COOH)

Ascorbic acid

2, 6-Dichloroindophenol (DCIP)(sodium salt)

Metaphosphoric acid (HPO_3)

Sodium bicarbonate (NaHCO_3)

Hazards

Corrosive

Corrosive

Preparing of Reagents

- **Ascorbic acid standard solution** (prepare only at time of use)

Accurately weigh (on an analytical balance) approximately 50 mg ascorbic acid (preferably U.S. Pharmacopeia (USP) Ascorbic Acid Reference Standard). Record this weight. Transfer to a 50-mL volumetric flask. Dilute to volume *immediately before use* with the metaphosphoric acid - acetic acid solution.

- Indophenol solution - dye

To 50 ml deionized distilled (dd) water in a 150-ml beaker, add and stir to dissolve 42 mg sodium bicarbonate, then add and stir to dissolve 50 mg 2, 6-dichloroindophenol sodium salt. Dilute mixture to 200 ml with dd water. Filter through fluted filter paper into an amber bottle. Close the bottle with a stopper or lid and store refrigerated until used.

- Metaphosphoric acid-acetic acid solution

To a 250 ml beaker, add 100 ml dd water then 20 ml acetic acid. Add and stir to dissolve 7.5 g metaphosphoric acid. Dilute mixture to 250 ml with distilled water. Filter through fluted filter paper into a bottle. Close the bottle with a stopper or lid and store refrigerated until used.

- Orange juice samples

Use products processed and packaged in various ways (e.g., canned, reconstituted frozen concentrate, fresh squeezed, not-from-concentrate). Filter juices through cheesecloth to avoid problems with pulp when pipetting. Record from the nutrition label for each product the percent of the Daily Value for Vitamin C.

Hazards, Precautions, and Waste Disposal

Preparation of reagents involves corrosives. Use appropriate eye and skin protection. Otherwise, adhere to normal laboratory safety procedures. Waste likely may be put down the drain using a water rinse.

Procedure**Standardization of Dye**

1. Pipette 5 ml metaphosphoric acid-acetic acid solution into each of three 50-ml Erlenmeyer flasks.
2. Add 2.0 ml ascorbic acid standard solution to each flask.
3. Using a funnel, fill the buret with the indophenol solution (dye) and record the initial buret reading.
4. Place the Erlenmeyer flask under the tip of the buret. Slowly add indophenol solution to standard ascorbic acid solution until a light but distinct rose-pink color persists for > 5 s (takes about 15-17 ml). Swirl the flask as you add the indophenol solution.
5. Note final buret reading and calculate the volume of dye used.
6. Repeat steps 3-5 for the other two standard samples. Record the initial and final buret readings and calculate the volume of dye used for each sample.
7. Prepare blanks: Pipette 7.0 ml metaphosphoric acid-acetic acid solution into each of three 50-ml Erlenmeyer flasks. Add to each flask a volume of distilled water approximately equal

to the volume of dye used above (i.e., average volume of dye used to titrate three standard samples).

8. Titrate the blanks in the same way as steps 3-5 above. Record initial and final buret readings for each titration of the blank, and calculate the volume of dye used.

Analysis of Juice Samples

1. Pipet into each of three 50 ml Erlenmeyer flasks 5 mL metaphosphoric acid-acetic acid solution and 2 ml orange juice.
2. Titrate each sample with the indophenol dye solution (as you did in steps 3-5 above) until a light but distinct rose-pink color persists for >5 s.
3. Record the initial and final readings and calculate the difference to determine the amount of dye used for each titration.

Calculations

1. Using the data obtained in standardization of the dye, calculate the titer using the following formula:

$$\text{Titer} = F = \frac{\text{mg ascorbic acid in volume of standard solution titrated}^{**}}{[(\text{average ml dye used to titrate standards}) - (\text{average ml dye used to titrate blank})]} \quad \text{Eq. 5.19}$$

$^{**}\text{mg ascorbic acid in volume of standard solution titrated} = (\text{mg of ascorbic acid}/50 \text{ ml}) \times 2 \text{ ml}$

Eq. 5.20

2. Calculate the ascorbic acid content of the juice sample in mg/ml, using the equation that follows and the volume of titrant for each of your replicates. Calculate the mean and standard deviation of the ascorbic acid content for your juice (in mg/ml). Obtain from other lab members the mean ascorbic acid content (in mg/ml) for other types of juice. Use these mean values for each type of juice to express the Vitamin C content of the juice samples as milligrams ascorbic acid/100 ml.

$$\text{mg ascorbic acid/ml} = (X - B) \times (F/E) \times (V/Y) \quad \text{Eq. 5.21}$$

where:

X = ml for sample titration

B = average ml for sample blank titration

F = titer of dye (= mg ascorbic acid equivalent to 1.0 ml indophenol standard solution)

E = ml assayed (= 2 ml)

V = volume of initial assay solution (= 7 ml)

Y = volume of sample aliquot titrated (= 7 ml)

Example calculation:

Weight of ascorbic acid used = 50.2 mg

Average volume of titrant used:

Ascorbic acid standards = 15.5 ml

Blanks = 0.10 ml

Volume of titrant used for orange juice sample = 7.1 ml

$$\text{Titer} = F = \frac{[(50.2 \text{ mg} / 50 \text{ ml}) 2 \text{ ml}]}{(15.5 \text{ ml} - 0.10 \text{ ml})}$$

= 0.130 mg/ml

mg ascorbic acid/mL = (7.1 ml – 0.10 ml) × (0.130 mg/2 ml) × (7 ml/7 ml) = 0.455 mg/ml

0.455 mg/ml = 45.4 mg/100 ml

Vitamin A

Vitamin A is **sensitive** to ultraviolet (UV) light, air (and any prooxidants, for that matter), high temperatures, and moisture. Therefore, steps must be taken to avoid any adverse changes in this vitamin due to such effects. Steps include using low actinic glassware, nitrogen, and/or vacuum, as well as avoiding excessively high temperatures. The addition of an antioxidant at the onset of the procedure is highly recommended.

High-performance liquid chromatographic (HPLC) methods are considered the only acceptable methods to provide accurate food measurements of vitamin A activity.

Principle. The test sample is saponified with ethanolic KOH, vitamin A (retinol) is extracted into organic solvent and then concentrated and determined by HPLC on a silica column.

Sample Saponification

Transfer 40 ml of ready-to-use formula or fluid milk to a 100 ml digestion flask containing a stirring bar. For saponification, add 10 ml of ethanolic pyrogallol solution (i.e., 2% (w/v) pyrogallol in 95% ethanol) and 40-ml ethanolic KOH (i.e., 10% (w/v) KOH in 90% ethanol). Wrap the flask in aluminum foil and stir at room temperature for 18 hr, or at 70°C using the reflux vessel. Dilute to volume with ethanolic pyrogallol solution.

Extraction of Digest

Pipet 3 ml of digestive into a 15 ml centrifuge tube and add 2 ml of deionized H₂O. Extract Vitamin A with 7 ml of hexane: diethyl ether (85:15, v/v). Repeat extraction 2X with 7 ml portions of extractant. After extractions, transfer the organic layer to a 25 ml volumetric flask.

Add 1 ml of hexadecane solution (i.e., 1-ml hexadecane in 100 ml hexane) and dilute to volume with hexane. Pipette 15 ml of diluted extract into a test tube and evaporate under nitrogen. Dissolve the residue in 0.5 ml of heptane.

Chromatography Parameters

Column	4.6 mm x 150 mm packed with 3- μ m silica (Apex 3- μ m silica)
Mobile Phase	Isocratic elution; heptane containing 2-propanol (1-5%, v/v)
Injection Volume	100 μ l
Detector	UV, 340 nm
Flow Rate	1-2 ml/min

The HPLC analysis of vitamin A in milk and milk-based infant formula, **AOAC Method 992.04, 50.1.02.**

Microfluorometric Method (AOAC Method 967.22, 45.1.15)

Principle. Some vitamins, such as riboflavin, are naturally fluorescent and may be estimated by measuring the degree of fluorescence of a food extract and comparing against a set of standards. Others may be converted to fluorescent derivatives and the same process undertaken, e.g. thiamine may be converted to the fluorescent thiochrome.

This method measures both **ascorbic acid** and **dehydroascorbic acid**. Ascorbic acid, following oxidation to dehydroascorbic acid, is reacted with **o-phenylenediamine** to produce a **fluorescent quinoxaline compound**.

Ultrasonic Milk Analyzers

Milk quality control is the use of approved tests to ensure the application of approved practices, standards and regulations concerning the milk and milk products. The tests are designed to ensure that milk products meet accepted standards for chemical composition and purity as well as levels of different micro-organisms.

Ultrasonic milk analyzers are in operation and they make an accurate and reliable analysis of Cow, Sheep, Buffalo, UHT milk, Cream 45% and other milk liquid products. They measure milk parameters Fat, SNF, Density, Protein, Lactose, Added water, Milk sample temperature, Freezing point, Salts, and Total solids. The results of the rapid analysis can give the decision to receive or reject the milk supplier. External Printer with the capability to print out the results of the measurements.

Principle

- Ultrasonic material analysis is based on a simple principle of physics: the motion of any wave will be affected by the medium through which it travels. Thus, changes in one or more of four

easily measurable parameters associated with the passage of a high frequency sound wave through a material **transit time, attenuation, scattering, and frequency content** can be correlated with certain compositional parameters of milk.

- The Milk Analyzer launches a high frequency sound wave by exciting the ultrasonic transducer with a continuous wave impulse. The sound wave travels through the milk. An amplitude theory implemented in the milk Analyzer software **predicts** the magnitude of each of the scattering and absorption mechanisms.
- The movement of the particles (i.e. fat, solids not fat, etc.) relative to the continuous phase causes visco-inertial losses as the sound wave propagates through the sample. Drag between the liquid and the particles causes sound energy to be lost as **heat**.
- The **velocity** of the ultrasonic pulse and the temperature change of the sample are precisely measured.
- The **sound velocity and temperature** are directly correlated to the particulates in the sample, the milk Analyzer can provide reliable analyses of critical components such as **fat and solids not fat** by accurately assessing changes in these parameters. Other characteristics, such as protein, added water and freezing point, are calculated based upon the percentage of components measured using an exact **mathematical formula**.



Fig. 5.26 Ultrasonic milk analyzer instrument, sample volume 20 ml and test time 40 sec.

Chapter 6

Gel Electrophoresis in Food Analysis

Objectives

- After reading this chapter, you should be able to:
 - Recognizes principles of gel electrophoresis and applications in food analysis.
 - Practical separation of food proteins by electrophoresis.

Introduction

Electrophoretic techniques in gels have been used in food analysis for various tasks, like cultivar and species control of raw material, for developing and monitoring food preservation and technology processes, and uncovering illegal mixtures or addition of certain components in food products. They can be used for both qualitative and quantitative aspects. Depending on the analytical task and the substrate to be analyzed, different separation methods are employed, such as basic or acidic zone electrophoresis, SDS electrophoresis, and isoelectric focusing. Gel electrophoretic techniques do not require a high instrumental effort and they are relatively easy to carry out.

Principles

Electrophoresis is defined as the **migration of charged molecules** in a solution through an **electrical field**. Anions, which are negatively charged, migrate towards the anode (the positive electrode) whilst cations, being positively charged, migrate towards the negatively charged cathode. If a mixture of ions is placed at the center of a suitable medium and an electric potential is applied to the medium, the ions will separate as they move towards their respective electrodes. The degree of separation depends on a number of factors, including the charge per unit mass for each ion. This in turn is influenced by the pH of the medium and its ionic strength. A decrease in pH makes ions more positive, an increase in pH makes them more negative, and increasing the ionic strength suppresses the charges on the ions.

Applications of Gel Electrophoresis in Food Analysis

Electrophoretic techniques can be used as one step in a **purification** process or to aid in the biochemical characterization of a protein. Commercially available preparative

electrophoresis units are used to purify large quantities of protein. Alternatively, small quantities of protein can be eluted and collected from electrophoresis gels using electroelution techniques. Electrophoresis can also be used to determine the purity of a protein extract. Electrophoresis is often used to determine the **protein composition** of a food product. For example, differences in the protein composition of soy protein concentrates and whey protein concentrates produced by different separation techniques can be detected.

Separation of Proteins by Electrophoresis

Following separation by electrophoresis, the various bands are **identified**. This is commonly done by the use of dyes, which allows the qualitative identification of protein groups but may also be adapted for quantitative estimation through the use of instruments that measure the degree of colour produced. Various support media may be used for electrophoretic separations and include:

- (i) **Paper**. This is inexpensive but suffers from some mixing between zones due to the adsorption of molecules on the cellulose, and often requires a considerable time period for separation to be completed.
- (ii) **Agar gel**. The ready availability of this medium, the speed of separation and its transparent nature which allows photometric scanning for quantitative purposes, makes this a popular choice in much electrophoretic work.
- (iii) **Polyacrylamide gel**. As with agar gel, this is transparent, facilitating scanning in the visible and ultraviolet ranges. Additionally, its pore size is controllable, allowing more efficient separation based on the sizes and shapes of molecules as well as charge.

Polyacrylamide Gel Electrophoresis

The most common type of electrophoresis performed with proteins is **zonal electrophoresis** in which proteins are **separated** from a complex mixture into **bands** by migration in aqueous buffers through a solid polymer matrix called a gel. Polyacrylamide gels are the most common matrix for zonal electrophoresis of proteins, although other matrices such as starch and agarose may be used.

Gel matrices can be formed in glass tubes or as slabs between two glass plates. Separation depends on the friction of the protein within the matrix and the charge of the protein molecule as described by the following equation:

$$\text{Mobility} = \frac{(\text{Applied voltage}) (\text{Net charge on molecule})}{\text{Friction of the molecule}}$$

Eq. 6.1

Proteins are positively or negatively charged, depending on pH of solution and their iso electric point (pI).

The isoelectric point (pI) is defined as the pH at which a protein has **no net charge** in solution. Proteins aggregate and **precipitate** at their pI because there is no electrostatic repulsion between molecules. **A protein is negatively charged if solution pH is above its pI, whereas a protein is positively charged if solution pH is below its pI.** The magnitude of the charge and applied voltage will determine how far a protein will migrate in an electrical field. The higher the voltage and stronger the charge on the protein, the greater the migration within the electrical field.

Molecular size and shape, which determine the Stokes radius of a protein, also determine migration distance within the gel matrix. Mobility of proteins decreases as molecular friction increases due to an increase in Stokes radius; thus, smaller proteins tend to migrate faster through the gel matrix. Similarly, a decrease in pore size of the gel matrix will decrease mobility.

In non-denaturing or native electrophoresis, proteins are separated in their native form based on **charge, size, and shape** of the molecule.

Another form of electrophoresis commonly used for separating proteins is **denaturing electrophoresis**. Polyacrylamide gel electrophoresis (PAGE) with an anionic detergent, sodium dodecyl sulfate (SDS), is used to separate protein subunits by size. Proteins are solubilized and dissociated into subunits in a buffer containing SDS and a reducing agent. Reducing agents, such as mercaptoethanol or dithiothreitol, are used to reduce disulfide bonds within a protein subunit or between subunits. Proteins bind SDS, become negatively charged, and are separated based on size alone.

Procedures

power supply and electrophoresis apparatus containing the polyacrylamide gel matrix and two buffer reservoirs are necessary to perform a separation. A representative slab gel and electrophoresis unit is shown in **Figure 6.1**.

- The power supply is used to make the electric field by providing a source of constant current, voltage, or power.
- The electrode buffer controls the pH to maintain the proper charge on the protein and conducts the current through the polyacrylamide gel. Commonly used buffer systems include an anionic tris-(hydroxymethyl)aminomethane buffer with a resolving gel at pH 8.8 and a cationic acetate buffer at pH 4.3.

- The polyacrylamide gel matrix is formed by **polymerizing** acrylamide and a small quantity (usually 5% or less) of the cross-linking reagent, *N,N* - methylenebisacrylamide, in the presence of a catalyst, tetramethylethylenediamine (TEMED), and source of free radicals, ammonium persulfate, as illustrated in **Figure 6.2**. Gels can be made in the laboratory or purchased precast.

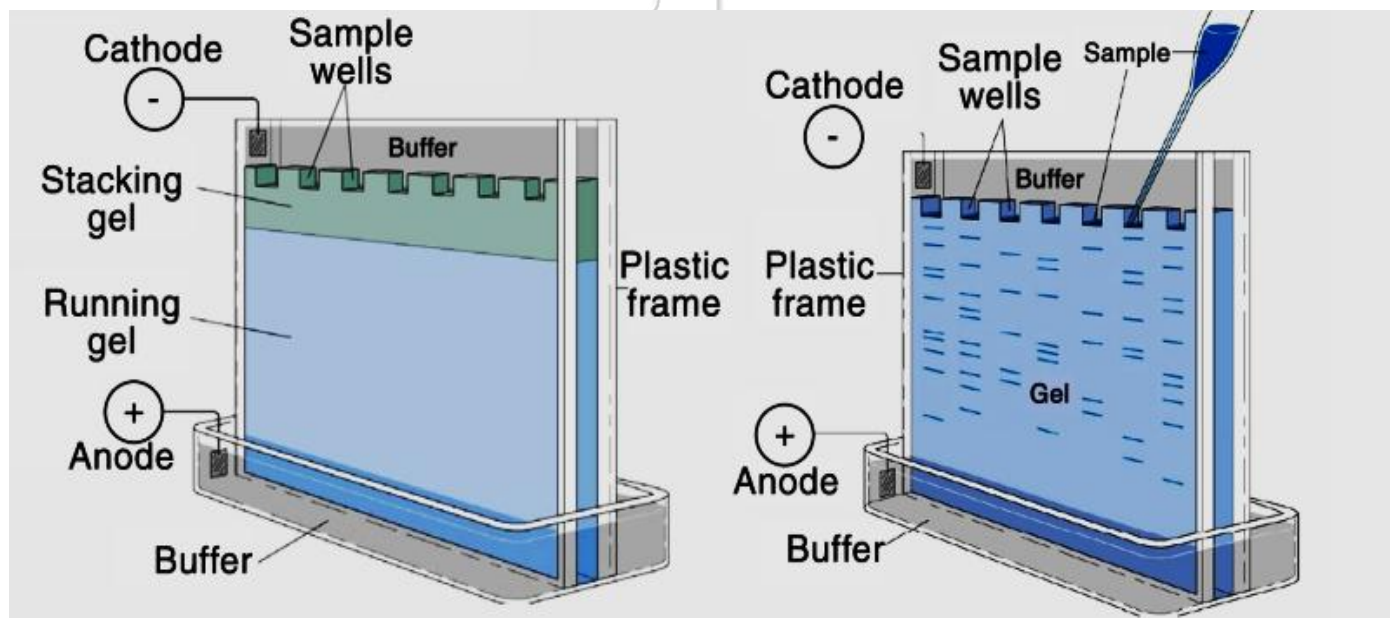


Fig. 6.1 Schematic of a slab gel electrophoresis unit

Acrylamide

***N,N*-methylene-
bis-acrylamide**

Polymer

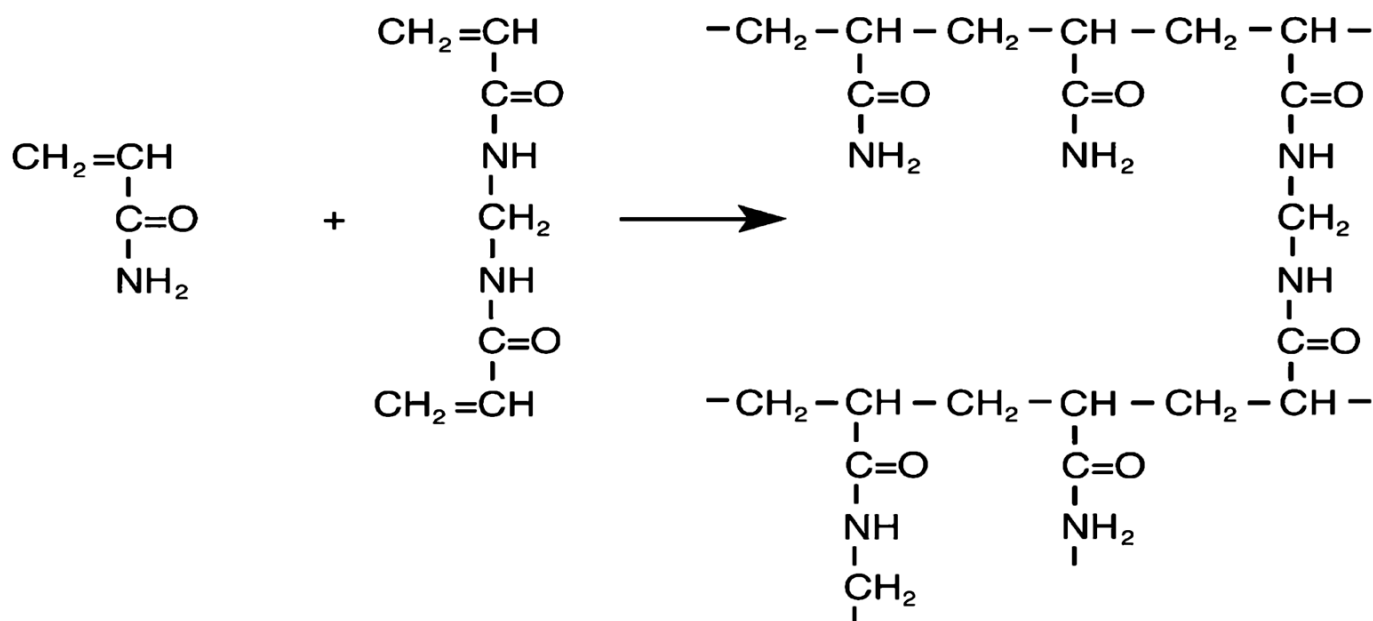


Fig. 6.2 Free radical polymerization reaction of polyacrylamide.

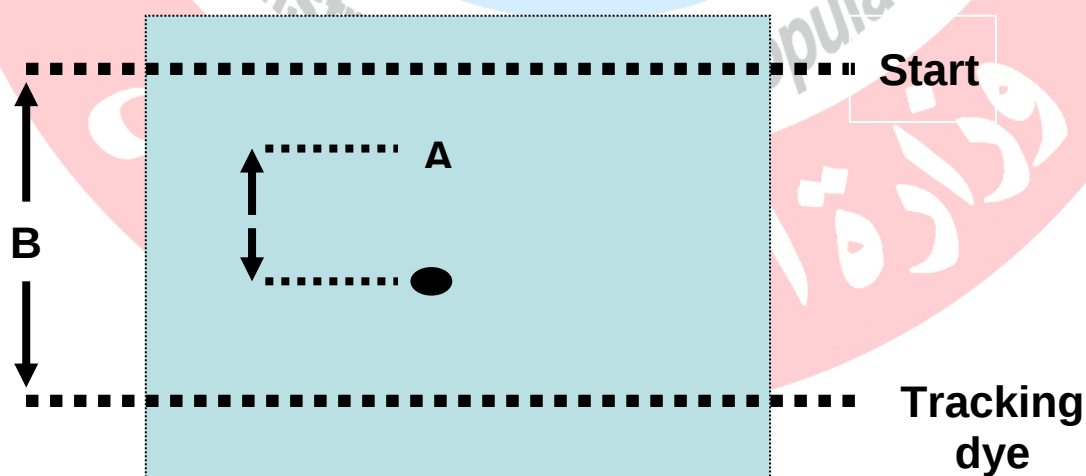
A discontinuous gel matrix is usually used to improve resolution of proteins within a complex mixture. The discontinuous matrix consists of a **stacking gel** with a large pore size (usually 3-4% acrylamide) and a **resolving gel** of a smaller pore size. The stacking gel, as its name implies, is used to stack or concentrate the proteins into very narrow bands prior to their entry into the resolving gel. At pH 6.8, a voltage gradient is formed between the chloride (high negative charge) and glycine ions (low negative charge) in the electrode buffer, which serves to stack the proteins into narrow bands between the ions. Migration into the resolving gel of a different pH disrupts this voltage gradient and allows separation of the proteins into discrete bands.

Proteins are usually separated on resolving gels that contain 4-15% acrylamide. Acrylamide concentrations of 15% may be used to separate proteins with molecular mass below 50,000 dalton (Da). Proteins greater than 500,000 Da are often separated on gels with acrylamide concentrations below 7%.

To perform a separation, proteins in a buffer of the appropriate pH are loaded on top of the stacking gel. **Bromophenol blue tracking dye** is added to the protein solution. This dye is a small molecule that migrates ahead of the proteins and is used to monitor the progress of a separation. After an electrophoresis run, the bands on the gels are generally visualized using a protein stain such as **Coomassie Brilliant Blue** or **silver stain**. Specific enzyme stains or antibodies can be used to detect a protein.

The electrophoretic or **relative mobility (R_m)** of each protein band is calculated as:

$$R_m = \frac{\text{Distance protein migrated from start of resolving gel}}{\text{Distance between start of running gel and tracking dye}} \quad \text{Eq. 6.2}$$



$$R_m = \frac{A}{B}$$

SDS-PAGE is used in characterization protocols to determine subunit composition of a protein and to estimate subunit molecular mass. Molecular mass can usually be estimated within an error of $\pm 5\%$, although highly charged proteins or glycoproteins may be subject to a larger error. Molecular mass is determined by comparing R_m of the protein subunit with R_m of **protein standards** of known molecular mass (**Figure 6.3**). Commercially prepared protein standards are available in several molecular mass ranges. To prepare a standard curve, logarithms of protein standard molecular mass are plotted against their corresponding R_m values. The molecular mass of the unknown protein is determined from its R_m value using the standard curve.

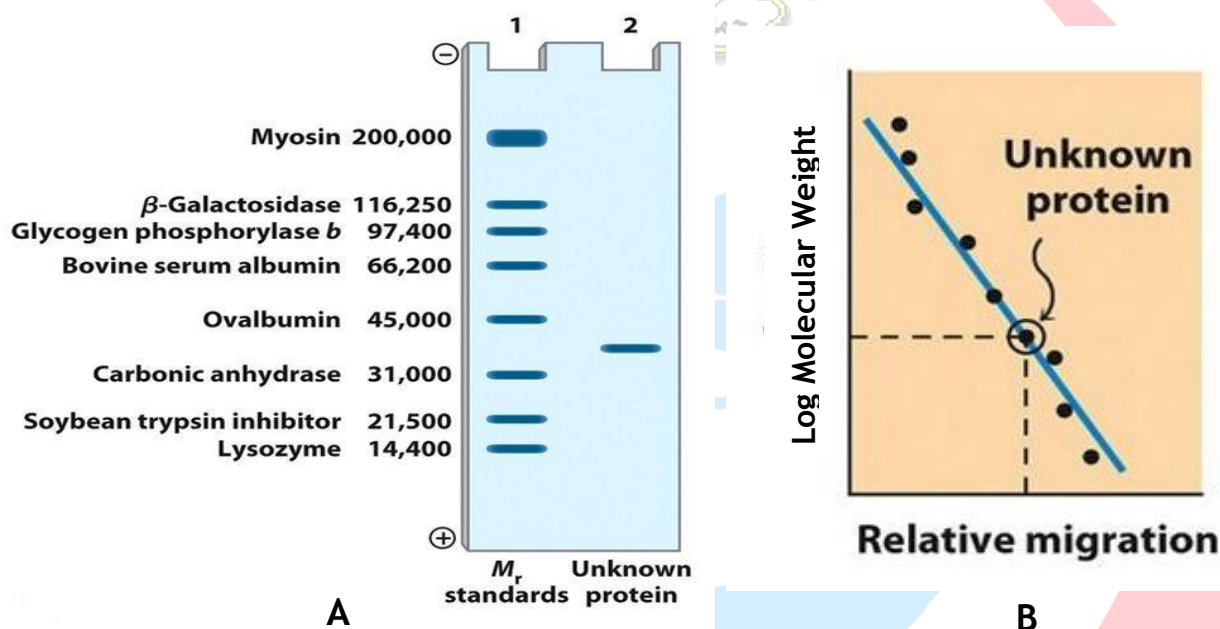


Fig. 6.3 Use of SDS-PAGE to determine the molecular mass of a protein. (a) Separation of molecular mass standards and the unknown protein. (b) Standard curve for estimating protein molecular mass.

In food science, electrophoresis plays an important role in the study of proteins. With foods such as milk, for example, the proteins present may have quite different structures and properties or may be very similar. Thus caseins, the major group of proteins present in milk, may be readily separated from the whey proteins based on solubility differences alone, but to identify and quantify individual casein fractions requires their electrophoretic separation.

Isoelectric Focusing

Principle. Isoelectric focusing is a modification of electrophoresis, in which proteins are separated by charge in an electric field on a gel matrix in which a pH gradient has been generated using ampholytes. Proteins are focused or migrate to the location in the gradient at which pH equals the pI of the protein. Resolution is among the highest of any protein separation technique and can be used to separate proteins with pIs that vary less than 0.02 of a pH unit.

Procedure

A pH gradient is formed using **ampholytes**, which are small polymers (molecular mass of about 5000 Da) containing both positively and negatively charged groups. An ampholyte mixture is composed of thousands of polymers that exhibit a range of pH values. Ampholytes are added to the gel solution prior to polymerization. After the gel is formed and a current applied, the ampholytes migrate to produce the pH gradient; negatively charged ampholytes migrate toward the anode while positively charged ampholytes migrate toward the cathode. Ampholyte mixtures are available that cover a narrow pH range (2-3 units) or a broad range (pH 3- 10) and should be selected for use based on properties of the proteins to be separated.

Applications

Isoelectric focusing is the method of choice for determining the isoelectric point of a protein and is an excellent method for determining the **purity** of a protein preparation. For example, isozymes of polyphenol oxidase and other plant and animal proteins are identified using isoelectric focusing. Isoelectric focusing is used to differentiate closely related fish species based on protein patterns.

Isoelectric focusing and SDS-PAGE can be combined to produce a two-dimensional electrophoretogram that is extremely useful for separating very complex mixtures of proteins. This technique is called **two-dimensional electrophoresis**. Proteins are first separated in tube gels by isoelectric focusing. The tube gel containing the separated proteins is then placed on top of an SDS-PAGE slab gel, and proteins are separated. Thus, proteins are separated first on the basis of charge and then according to size and shape. Over 1000 proteins in a complex mixture have been resolved using this technique.

Capillary Electrophoresis

Capillary electrophoresis (CE) is a technique utilizing a silica capillary tube normally filled with a buffer solution, although for certain purposes this may be internally coated or filled with a gel matrix. The ends of the tube are placed in electrolyte reservoirs and a high voltage is applied across these two solutions. This causes migration of charged species and the bands produced are detected and measured online, providing immediate results as with a

chromatogram. The process allows separations that would take hours with conventional electrophoretic techniques to be achieved in minutes with CE.

Procedure

A schematic diagram of a capillary electrophoresis system is shown in **Figure 6.4**. A capillary electrophoresis system comprises a capillary column, power supply, detector, and two buffer reservoirs. The sample is introduced into the inlet side of the capillary tube by simply replacing the inlet buffer reservoir with the sample solution and applying low pressure or voltage across the capillary until the desired volume of sample has been loaded onto the column. Capillaries are composed of fused silica with internal diameters that commonly range from 25 to 100 μm . Column length varies from a few centimeters to 100 cm. High electric fields (100-500V/cm) can be used as the narrow columns dissipate heat very effectively, allowing for short run times of 10-30 min.

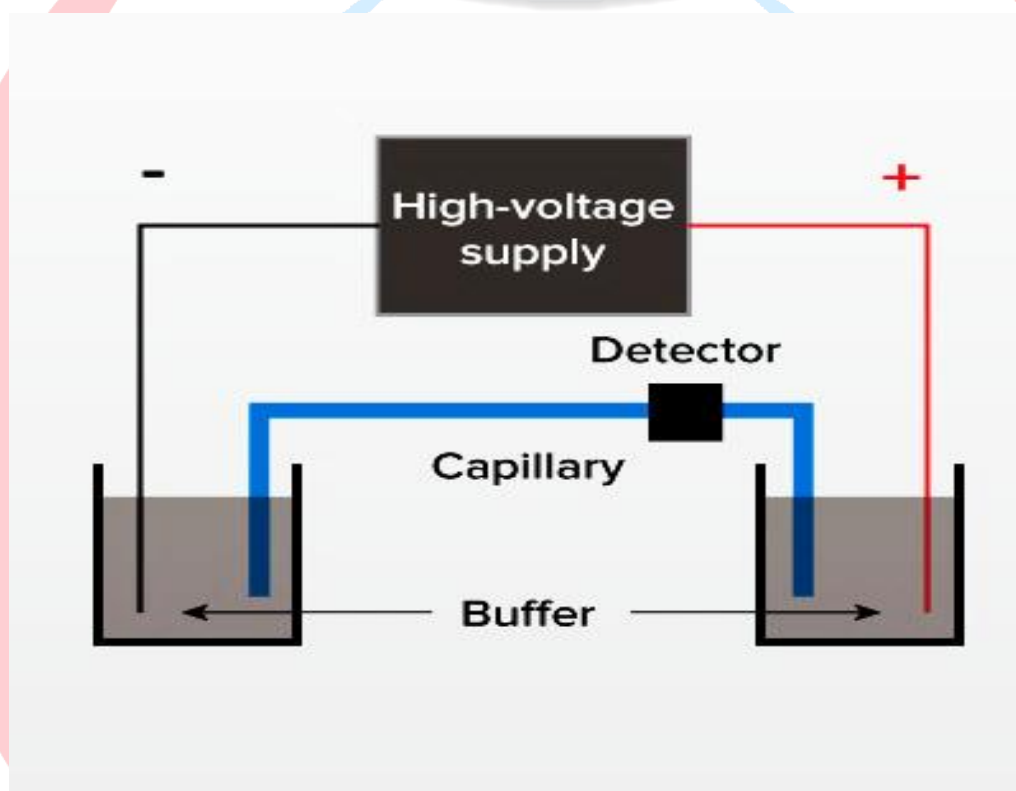


Fig. 6.4 Schematic of a capillary electrophoresis system.

At the end of a run protein bands are not visualized by staining as in conventional electrophoresis. Instead, protein bands are detected on the column as they migrate past a detector. The detectors are similar to those used in HPLC described in **chap.8**. Ultraviolet (UV)-visible detectors are most common.

Capillary zone electrophoresis or **free solution electrophoresis** is much like native PAGE, except proteins are separated in free solution inside capillary tubes filled with buffer of the desired pH. Diffusion is prevented within the narrow diameter of the capillaries, so a gel matrix is not required. In capillary zone electrophoresis, electroosmotic flow also influences the separation of proteins within capillary tubes. The negatively charged fused silica capillary wall [containing silanol groups (SiO^-)] attracts positively charged ions (**cations**) from the buffer to form a double ion layer at the interface between the capillary column wall and the buffer. When the electric field is applied, the cations forming the double layer are attracted toward the cathode and “pull” other molecules (independent of charge) in the same direction. Thus, in free solution capillary electrophoresis, cations, anions, and uncharged molecules can be separated in a single run.

Electroosmotic flow can be controlled by changing the pH or ionic strength of the buffer to alter the charge on the capillary wall and change the rate of protein migration. Capillary zone electrophoresis is used for a variety of applications, including the **fractionation** of milk, cereal, soybean, and muscle proteins.

SDS capillary gel electrophoresis techniques can be used to separate proteins by size and to determine molecular mass. In this technique proteins are **denatured** and dissociated in the presence of SDS and a reducing agent, then fractionation occurs in polyacrylamide gel-filled capillaries of specific pore sizes. Alternatively, linear polymers, such as methyl cellulose, dextrans, or polyethylene glycol, are added to the buffer within the capillary in a technique called **dynamic sieving capillary electrophoresis**. These entangled polymers act like the pores of the polyacrylamide gel to slow migration of the larger proteins and allow **separation by size**.

Chapter 7

Spectroscopy Instruments and Applications in Food Analysis

Objectives

- After reading this chapter, you should be able to:
 - Define the terms related to electromagnetic radiation.
 - Recognize spectroscopy instruments theory and applications in the food analysis.

Introduction

Spectroscopy deals with the production, measurement, and interpretation of spectra arising from the **interaction of electromagnetic radiation with matter**. There are many different spectroscopic methods available for solving a wide range of analytical problems. The methods differ with respect to the species to be analyzed (such as molecular or atomic spectroscopy), the type of radiation-matter interaction to be monitored (such as absorption, emission, or diffraction), and the region of the electromagnetic spectrum used in the analysis. Spectroscopic methods are very informative and widely used for both quantitative and qualitative analyses. Spectroscopic methods based on the absorption or emission of radiation in the **ultraviolet (UV)**, **visible (Vis)**, **infrared (IR)**, and **radio (nuclear magnetic resonance, NMR)** frequency ranges are most commonly encountered in traditional food analysis laboratories. **Figure 7.1** shows the electromagnetic spectrum.

The interaction of the light (electromagnetic radiation) with a substance and the subsequent energy transfer ends with three main processes namely:

Absorption:

The process by which the energy of the light (in the form of photons) is transferred to the atom or molecule raising them from the ground state to an excited state.

Fluorescence:

The absorbed energy is rapidly lost to the surroundings by collisions within the system and relax back to the ground state. Sometimes the energy is not lost in this way but is re-emitted a few milliseconds later, which is referred to as fluorescence.

Emission:

If the substances (atoms or molecules) are heated to high temperatures (in a flame or in an electric discharge) the electrons are excited to higher energy levels. Later, they relax to the

ground state with the emission of radiation, the magnitude of which is more or less equivalent to absorbed energy.

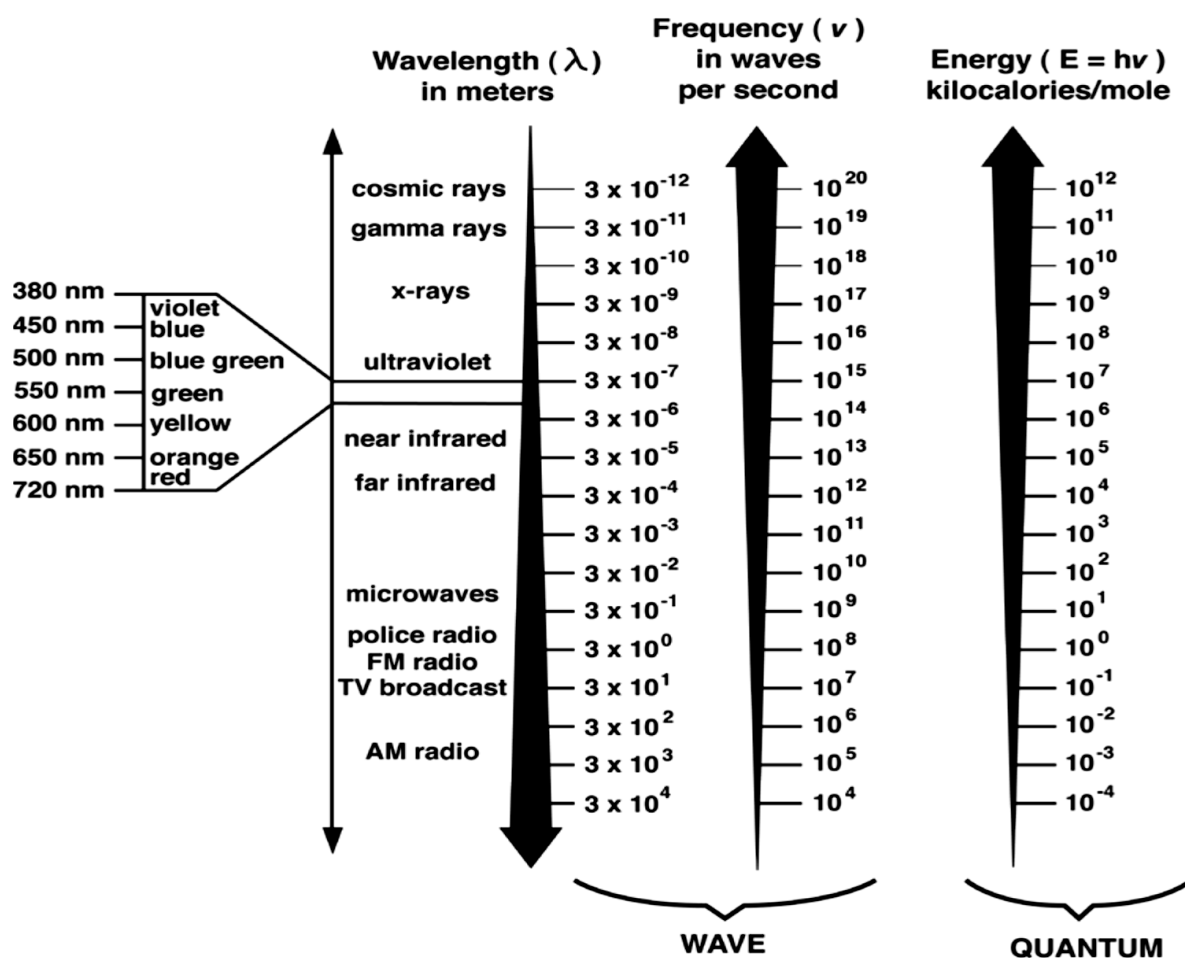


Fig. 7.1 The electromagnetic spectrum

Definitions associated with electromagnetic radiation and which relate to the wave patterns of the particular radiation include the following:

Wavelength (λ). This is the distance between successive wave peaks and is measured in nm (where 1 nm is equal to 10^{-9} m). Measurements are sometimes quoted in Angstrom units ($\text{\AA}$) where $1\text{\AA}$ is equal to 10^{-10} m; the $\text{\AA}$ is, however, not an acceptable SI unit.

Frequency (ν). This is the number of successive peaks passing a given point in 1 second. Frequency is related to wavelength by the equation:

$$\nu = 1/\lambda$$

Eq. 7.1

Wavenumber. This is defined as:

$$\text{Wavenumber (cm}^{-1}\text{)} = 1 / \text{wavelength (cm)} = 1 \times 10^7 / \text{wavelength (nm)}$$

Energy(E). For a particular wave form the energy of that radiation is given by:

$$E = h\nu = hc/\lambda$$

Eq. 7.2

where h = Planck's constant = 6.624×10^{-34} joule/s, ν = frequency of radiation, λ = wavelength, and c = velocity of light = 3.0×10^{10} cm/s.

Ultraviolet, Visible Spectroscopy

Spectroscopy in the ultraviolet-visible (UV-Vis) range is one of the most commonly encountered laboratory techniques in food analysis. Diverse examples, such as the quantification of macro components (total carbohydrate by the phenol-sulfuric acid method). Electromagnetic radiation in the UV-Vis portion of the spectrum ranges in wavelength from approximately 200-700 nm. The accessible **UV range** for common laboratory analyses runs from 200 to 350 nm and the **Vis range** from 350 to 700 nm (Table 7.1). The UV range is colorless to the human eye, while different wavelengths in the visible range each have a characteristic color, ranging from violet at the short wavelength end of the spectrum to red at the long wavelength end of the spectrum. Spectroscopy utilizing radiation in the UV-Vis range may be divided into two general categories, **absorbance** and **fluorescence** spectroscopies, based on the type of radiation matter interaction that is being monitored. Each of these two types of spectroscopy may be subdivided further into **qualitative** and **quantitative** techniques. In general, quantitative absorption spectroscopy is the most common of the subdivisions within UV-Vis spectroscopy.

Table 7.1 Spectrum of visible radiation

Wavelength (nm)	Color	Complementary hue**
<380	Ultraviolet	
380-420	Violet	Yellow-green
420-440	Violet-blue	Yellow
440-470	Blue	Orange
470-500	Blue-green	Red
500-520	Green	Purple
520-550	Yellow-green	Violet
550-580	Yellow	Violet-blue
580-620	Orange	Blue
620-680	Red	Blue-green
680-780	Purple	Green
>780	Near infrared	

**Complementary hue refers to the color observed for a solution that shows maximum absorbance at the designated wavelength assuming a continuous spectrum "white" light source.

Basis of Quantitative Absorption Spectroscopy

The objective of quantitative absorption spectroscopy is to determine the **concentration** of analyte in a given sample solution. The determination is based on the measurement of the amount of light absorbed from a reference beam as it passes through the sample solution. In

some cases, the analyte may naturally absorb radiation in the UV-Vis range, such that the chemical nature of the analyte is not modified during the analysis. In other cases, analytes that do not absorb radiation in the UV-Vis range are chemically modified during the analysis, converting them to a species that absorbs radiation of the appropriate wavelength. In either case, the presence of analyte in the solution will affect the amount of radiation transmitted through the solution, and, hence, the relative transmittance or absorbance of the solution may be used as an index of analyte concentration.

In actual practice, the solution to be analyzed is contained in an absorption cell and placed in the path of radiation of a selected wavelength(s). The amount of radiation passing through the sample is then measured relative to a reference sample. The relative amount of light passing through the sample is then used to estimate the analyte concentration. The process of absorption may be depicted as in **Figure 7.2**. The radiation incident on the absorption cell, (I_0), will have significantly greater radiant power than the radiation exiting the opposite side of the cell, (I_t). The decrease in radiant power as the beam passes through the solution is due to the capture (absorption) of photons by the absorbing species. The relationship between the power of the incident and exiting beams typically is expressed in terms of either the transmittance or the absorbance of the solution.

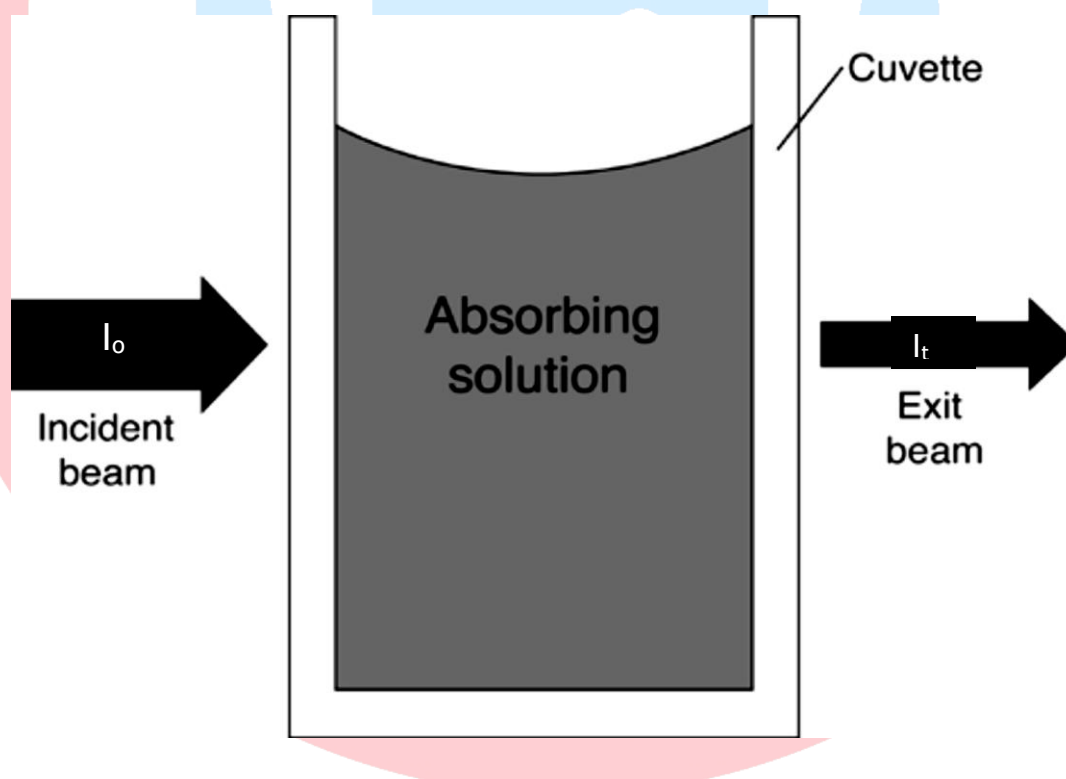


Fig. 7.2 Attenuation of a beam of radiation as it passes through a cuvette containing an absorbing solution

The **transmittance** (T) of a solution is defined as the ratio of I_t to I_o as given in Eq. 7.3
Transmittance also may be expressed as a percentage as given in Eq. 7.4.

$$\text{Transmittance } (T) = I_t / I_o \quad \text{Eq. 7.3}$$

$$\% \text{ Transmittance } (T) = I_t / I_o \times 100 \quad \text{Eq. 7.4}$$

Where:

I = radiant power of beam incident on absorption cell

I_t = radiant power of beam exiting the absorption cell

A second term used to describe the relationship between I_t and I_o is **absorbance** (A). Absorbance is defined with respect to T as shown in Eq. 7.5.

$$\text{Absorbance } (A) = -\log (T) = -\log (I_t / I_o) = \log (I_o / I_t) \quad \text{Eq. 7.5}$$

Absorbance is a convenient expression in that, under appropriate conditions, it is directly proportional to the concentration of the absorbing species in the solution. Note that based on these definitions for A and T , the absorbance of a solution *is not* simply unity minus the transmittance. In quantitative spectroscopy, the fraction of the incident beam that is not transmitted does not equal the solution's absorbance (A).

The relationship between the absorbance of a solution and the concentration of the absorbing species is known as Eq. 7.6.

$$A = \epsilon c l \quad \text{Eq. 7.6}$$

Where

ϵ = **molar absorption coefficient** of solute being measured ($\text{mole}^{-1} \text{cm}^{-1}$), is given as a constant and varies for each molecule, c = **concentration of solute** in solution (M , mM , mg/mL , $\%$), and l = **length of light path** (cm).

The Beer's law applies not only to colored solutions but also to solutions that may absorb other forms of radiation. Since the law is not a fundamental law of nature, but rather an experimental law, it only holds true under certain limiting conditions and may be subject to the **following errors**:

- (i) The difference between I_o and I_t is not totally a measure of absorbed radiation since some radiation is reflected and some is absorbed by the sample holder (cuvette) material.
- (ii) The solvent used for dissolving the sample may also absorb some radiation.
- (iii) The sample may undergo changes such as association, dissociation, etc.

- (iv) The wavelength of the incident light may not be strictly monochromatic and may be composed of too wide a wavelength range.
- (v) The law only holds true up to a limiting concentration.

Many of the potential errors indicated above may be reduced or eliminated by:

- (i) The use of blank samples.
- (ii) The use of cuvettes of appropriate quality and material for the analysis in question, e.g. glass cuvettes are generally superior to the cheaper, disposable plastic types, while for UV wavelengths, where glass absorbs strongly, quartz or fused silica material is required.
- (iii) Setting the wavelength to that of maximum absorption and thus the greatest sensitivity.

Quantitative spectroscopy is dependent on the analyst being able to accurately measure the fraction of an incident light beam that is absorbed by the analyte in a given solution. This apparently simple task is somewhat complicated in actual practice due to processes other than analyte absorption that also result in significant decreases in the power of the incident beam. A pictorial summary of reflection and scattering processes that will decrease the power of an incident beam is given in **Figure 7.3**. It is clear that these processes must be accounted for if a truly quantitative estimate of analyte absorption is necessary. In practice, a reference cell is used to correct for these processes. A **reference cell** is one that, in theory, exactly matches the sample absorption cell with the exception that it contains no analyte. Reference cells are often prepared by filling appropriate absorption cells with water. The reference cell is placed in the path of the light beam, and the power of the radiation exiting the reference cell is measured and taken as I_0 for the sample cell. This procedure assumes that all processes except the selective absorption of radiation by the analyte are equivalent for the sample and reference cells. The absorbance actually measured in the laboratory approximates (Eq.7.7).

$$A = \log (I_t \text{ Solvent} / I_t \text{ analyte solution}) = \log (I_0 / I_t)$$

Eq. 7.7

Where:

$I_t \text{ Solvent}$ = radiant power of beam exiting cell containing solvent (blank)

$I_t \text{ analyte solution}$ = radiant power of beam exiting cell containing analyte solution

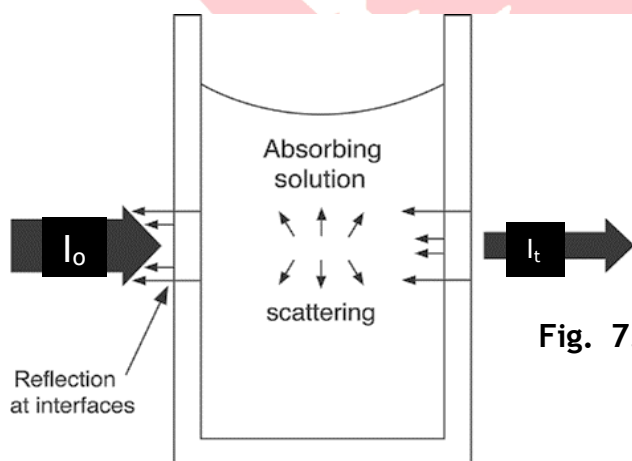


Fig. 7.3 Factors contributing to the attenuation of a beam of radiation as it passes through a cuvette containing an absorbing solution.

Procedural Considerations

The goal of many quantitative measurements is to determine the **concentration** of an analyte with optimum **precision and accuracy**, in a minimal amount of time, and at minimal cost. To accomplish this, it is essential that the analyst consider potential errors associated with each step in a particular assay.

Sample preparation schemes for absorbance measurements vary considerably. In the simplest case, the analyte-containing solution may be measured directly following homogenization and clarification. Except for special cases, homogenization is required prior to any analysis to ensure a representative sample. Clarification of samples is essential prior to taking absorbance readings in order to avoid the apparent absorption due to scattering of light by turbid solutions. The **reference solution** for samples in this simplest case will be the sample solvent, the solvent being water or an aqueous buffer in many cases.

A **sample-holding cell** or **cuvette** should be composed of a material that does not absorb radiation in the spectral region being used. Cells meeting this requirement for measurements in the **UV range** may be composed of **quartz or fused silica**. For the **Vis range** cells made of **silicate glass** are appropriate, and inexpensive plastic cells also are available for some applications. The dimensions of the cell will be important with respect to the amount of solution required for a measurement and with regard to the pathlength term used in **Beer's law**. A typical absorption cell is 1 cm² and approximately 4.5 cm long. The pathlength for this traditional cell is 1 cm, and the minimum volume of solution needed for standard absorption measurements is approximately 1.5 mL. Absorption cells with pathlengths ranging from 1 to 100 mm are commercially available.

Calibration or Standard Curves

The calibration curve is used to establish the relationship between analyte **concentration** and **absorbance**. This relationship is established experimentally through the analysis of a **series of samples of known analyte concentration**. The standard solutions are best prepared with the same reagents and at the same time as the unknown. The concentration range covered by the standard solutions must include that expected for the unknown. Typical calibration curves are depicted in **Figure 7.4**. Linear calibration curves are expected for those systems that obey Beer's law.

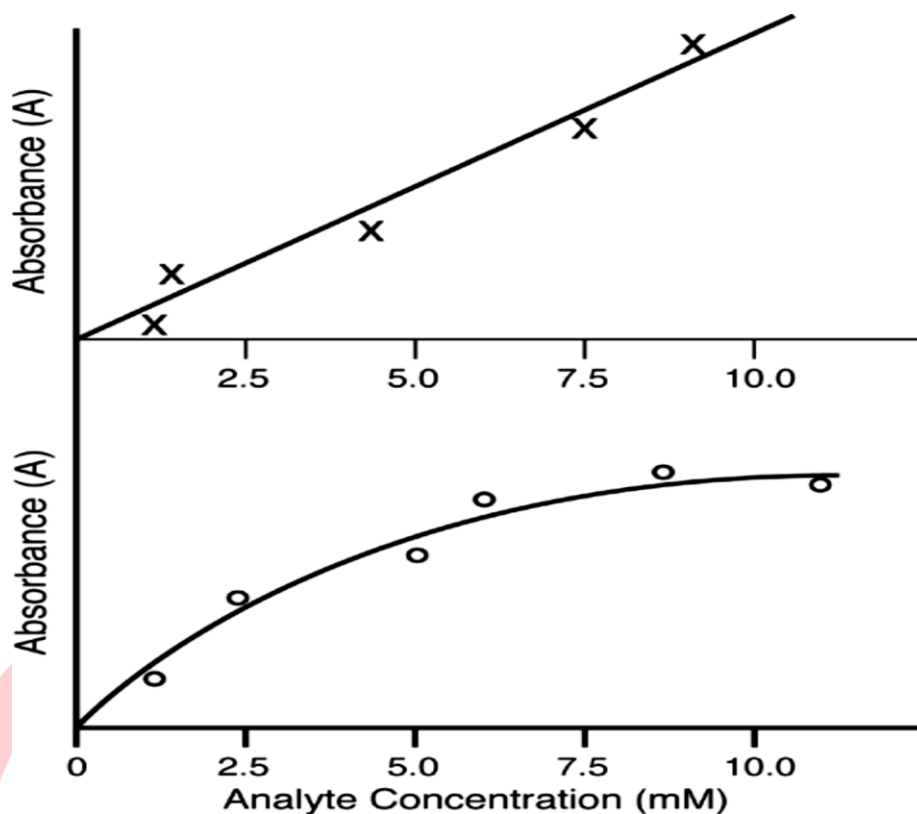


Fig. 7.4 Linear (a) and nonlinear (b) calibration curves typically encountered in quantitative absorption spectroscopy

Instrumentation

There are many variations of spectrophotometers available for UV-Vis spectrophotometry. Some instruments are designed for operation in only the visible range, while others encompass both the UV and Vis ranges. A basic spectrophotometer is composed of five essential components: the **light source**, the **monochromator**, the **sample/reference holder**, the **radiation detector**, and a **readout device**. A power supply is required for instrument operation. A schematic depicting component interrelationships is shown in **Figure 7.5**.

A. Light Source

Light sources used in spectrophotometers must continuously emit a strong band of radiation encompassing the entire wavelength range for which the instrument is designed. The most common radiation source for **Vis spectrophotometers** is the **tungsten filament lamp**. These lamps emit adequate radiation covering the wavelength region from 350 to 2,500 nm. Consequently, tungsten filament lamps also are employed in nearinfrared spectroscopy. The most common radiation sources for measurements in the **UV range** are **deuterium electrical-discharge lamps**. These sources provide a continuous radiation spectrum from approximately

160 nm through 375 nm. These lamps employ quartz windows and should be used in conjunction with quartz sample holders, since glass significantly absorbs radiation below 350 nm.

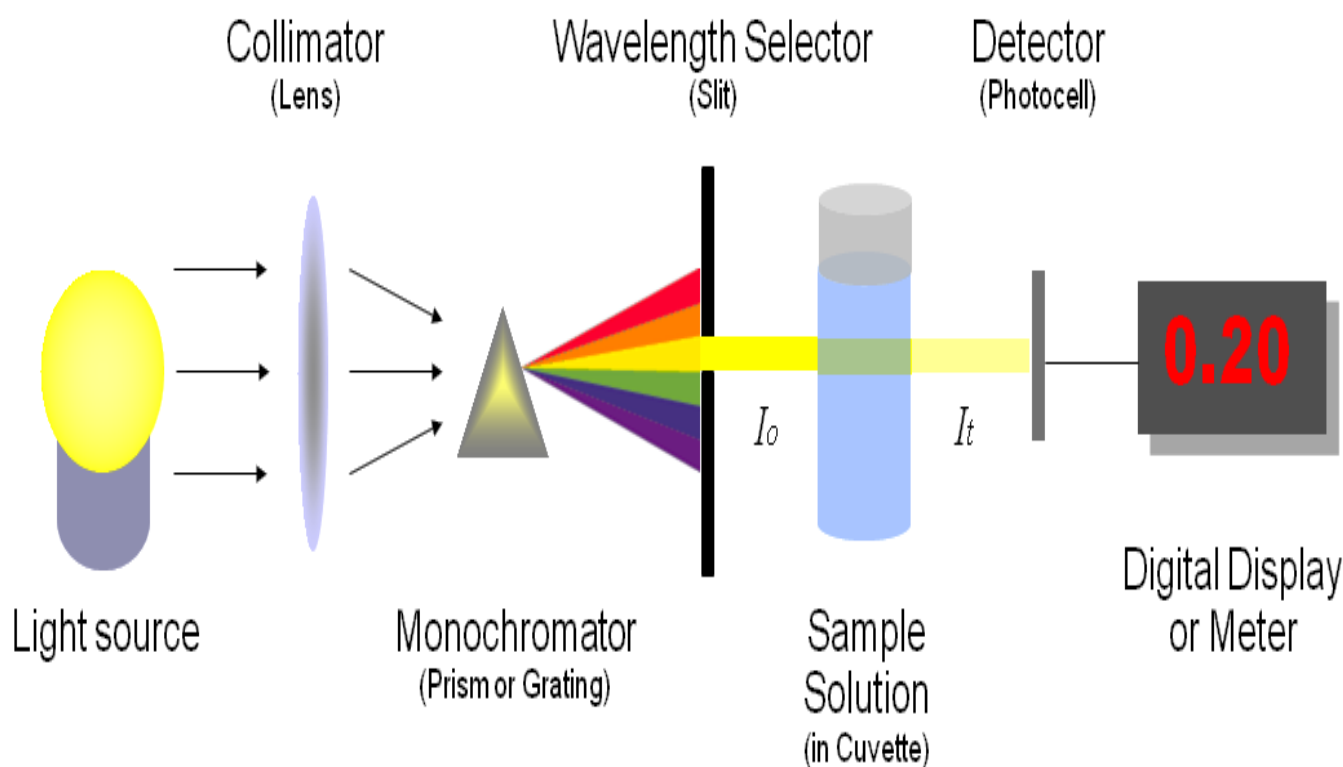


Fig. 7.5 Arrangement of components in a simple single-beam, UV-Vis absorption spectrophotometer

B. Monochromator

The monochromator is the component that functions to isolate the specific, narrow, continuous group of wavelengths to be used in the spectroscopic assay. The monochromator is so named because light of a single wavelength is termed **monochromatic**. Theoretically, polychromatic radiation from the source enters the monochromator and is dispersed according to wavelength, and monochromatic radiation of a selected wavelength exits the monochromator. In practice, light exiting the monochromator is not of a single wavelength, but rather it consists of a **narrow continuous band of wavelengths**. A typical monochromator is composed of **entrance and exit slits, concave mirror(s), and a dispersing element**.

Polychromatic light enters the monochromator through the entrance slit and is then culminated by a concave mirror. The culminated polychromatic radiation is then dispersed, dispersion being the physical separation in space of radiation of different wavelengths. The radiation of different wavelengths is then reflected from a concave mirror that focuses the different wavelengths of light sequentially along the focal plane. The radiation that aligns with the exit slit in the focal

plane is emitted from the monochromator. The radiation emanating from the monochromator will consist of a narrow range of wavelengths presumably centered around the wavelength specified on the wavelength selection control of the instrument.

C. Detector

In a spectroscopic measurement, the light transmitted through the reference or sample cell is quantified by means of a detector. The detector is designed to produce an **electric signal** when it is struck by **photons**. An ideal detector would give a signal directly proportional to the radiant power of the beam striking it, it would have a high signal-to-noise ratio, and it would have a relatively constant response to light of different wavelengths, such that it was applicable to a wide range of the radiation spectrum. There are several types and designs of radiation detectors currently in use. The most commonly encountered detectors are the **phototube**, the **photomultiplier tube**, and **photodiode detectors**. All of these detectors function by converting the **energy** associated with incoming photons into **electrical current**.

D. Readout Device

The signal from the detector generally is amplified and then displayed in a usable form to the analyst. Digital readouts express the signal as numbers on the face of a meter. There is an obvious requirement for signal processing between the analog output of the detector and the final digital display. The signal processor is capable of presenting the final readout in terms of either **absorbance** or **transmittance**. Many of the newer instruments include **microprocessors** capable of more extensive data manipulations on the digitized signal. For example, the readouts of some spectrophotometers may be in concentration units provided the instrument has been correctly calibrated with appropriate reference standards.

Instrument Design

The optical systems of spectrophotometers fall into one of two general categories: they are either single-beam or double-beam instruments.

In a **single-beam instrument**, the radiant beam follows only one path, that going from the source through the sample to the detector (**Figure 7.5**). When using a single-beam instrument, the analyst generally measures the transmittance of a sample after first establishing 100 % T , or I_0 , with a reference sample or blank. The blank and the sample are read sequentially since there is but a single light path going through a single cell-holding compartment.

In a **double-beam optical system**, the beam is split in time between the sample and reference cell, (**Figure 7.6**). In this design, the beam is alternately passed through the sample and

reference cells by means of a rotating sector mirror with alternating reflective and transparent sectors. The double-beam design allows the analyst to simultaneously measure and compare the relative absorbance of a sample and a reference cell. The advantage of this design is that it will compensate for deviations or drifts in the radiant output of the source since the sample and reference cells are compared many times per second. The disadvantage of the double-beam design is that the radiant power of the incident beam is diminished because the beam is split.

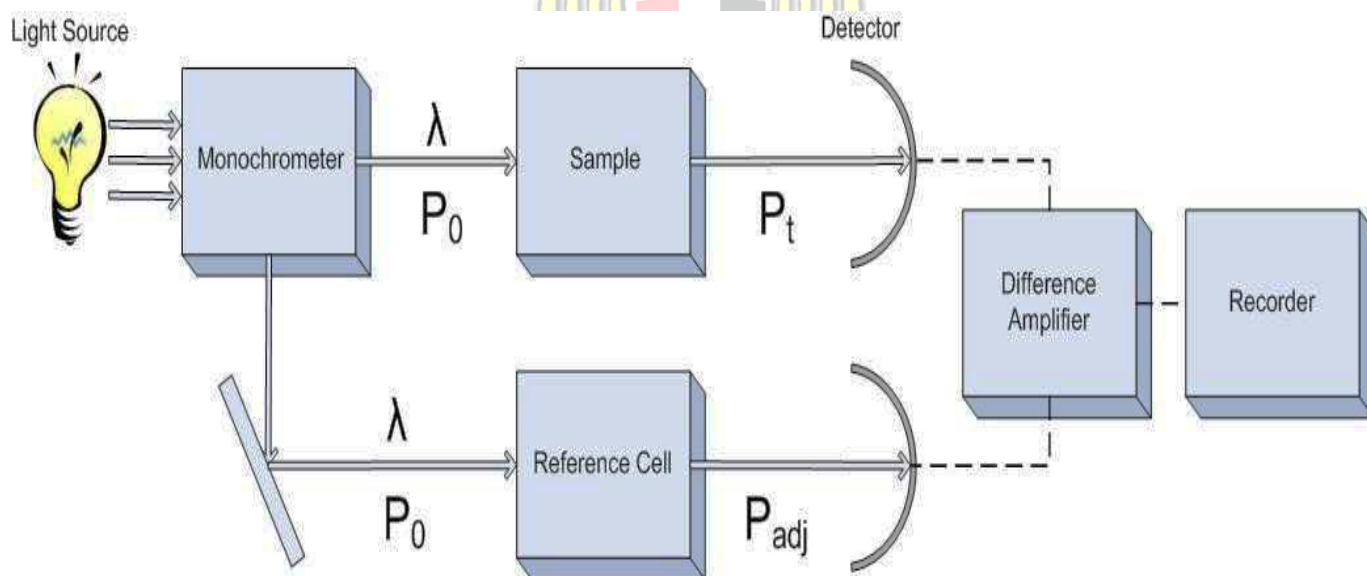


Fig. 7.6 Arrangement of components in a double-beam, UV-Vis absorption spectrophotometer

Computerized single-beam spectrophotometers now are available that claim to have the benefits of both the single- and double-beam designs. Their manufacturers report that previously troublesome source and detector drift and noise problems have been stabilized such that simultaneous reading of the reference and sample cell is not necessary. With these instruments, the reference and sample cells are read sequentially, and the data are stored, and then processed, by the associated computer.

Fluorescence Spectroscopy

In **fluorescence spectroscopy**, the signal being measured is the electromagnetic radiation that is **emitted** from the analyte as it relaxes from an **excited** electronic energy level to its corresponding ground state (**Figure 7.7**). The analyte is originally activated to the higher energy level by the absorption of radiation in the UV or Vis range. The processes of activation and deactivation occur simultaneously during a fluorescence measurement. For each unique molecular system, there will be an **optimum** radiation wavelength for sample excitation and

another, of longer wavelength, for monitoring fluorescence emission. The respective wavelengths for excitation and emission will depend on the chemistry of the system under study.

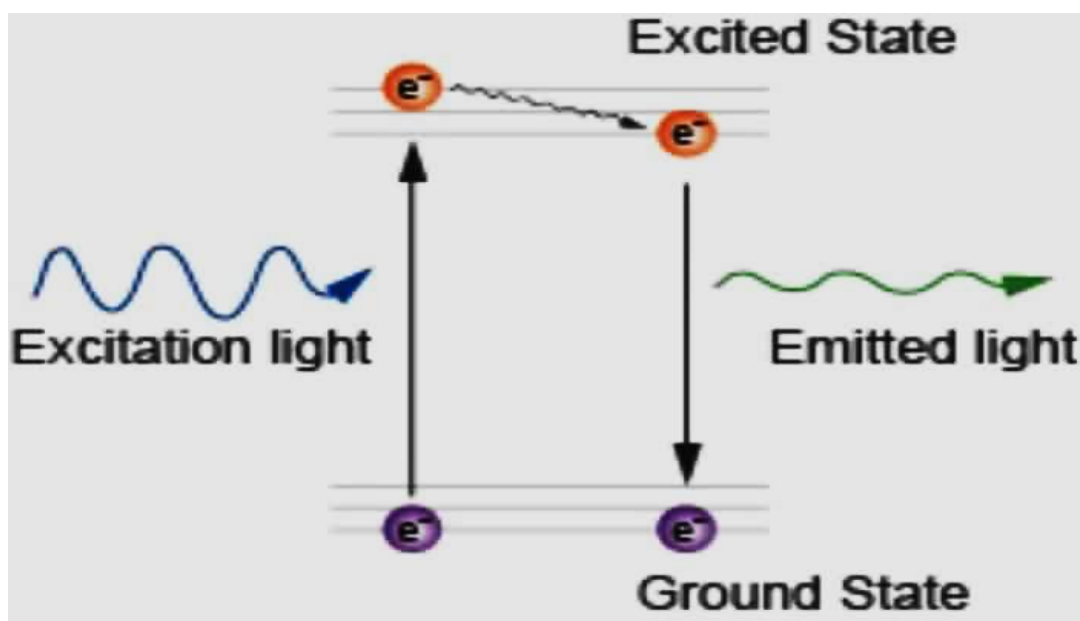


Fig. 7.7 Principles of Fluorescence

The instrumentation used in fluorescence spectroscopy is composed of essentially the same components as the corresponding instrumentation used in UV-Vis absorption spectroscopy.

However, there are definite differences in the arrangement of the optical systems used for the two types of spectroscopy (**Figure 7.8**). In fluorimeters and spectrofluorimeters, there is a need for two wavelength selectors, one for the **excitation beam** and one for the **emission beam**. In some simple fluorimeters, both wavelength selectors are filters such that the excitation and emission wavelengths are fixed. In more sophisticated spectrofluorimeters, the excitation and emission wavelengths are selected by means of grating monochromators. The photon detector of fluorescence instrumentation is generally arranged such that the emitted radiation that strikes the detector is traveling at an angle of 90° relative to the axis of the excitation beam. This detector placement minimizes signal interference due to transmitted source radiation and radiation scattered from the sample.

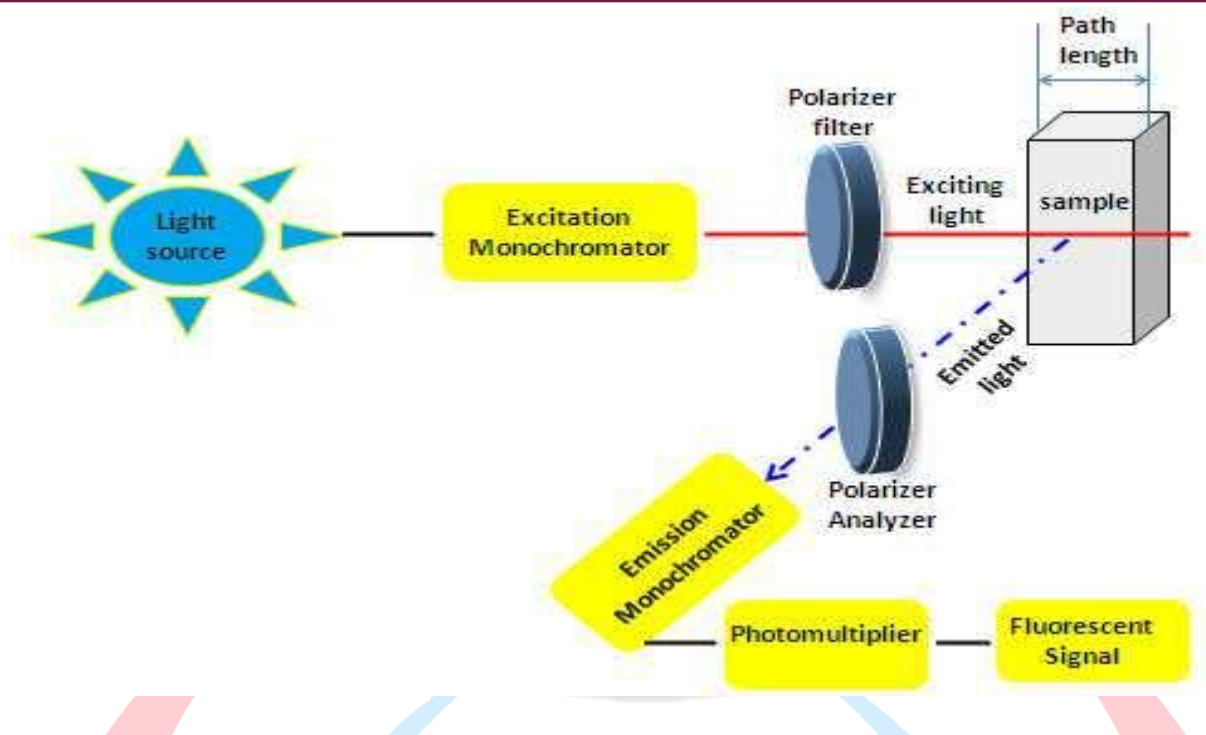


Fig.7.8 Arrangement of components in a fluorescence spectrophotometer

Infrared Spectroscopy

Infrared (IR) spectroscopy refers to measurement of the absorption of different frequencies of IR radiation by foods or other solids, liquids, or gases.

Principle

The IR absorption spectrum can be used to identify the molecule as a 'fingerprint' of a molecule with covalent bonds. Qualitative and quantitative identification of organic and inorganic compounds is a primary use of IR spectroscopy. **Infrared radiation** is electromagnetic energy with wavelengths (λ) longer than visible light but shorter than microwaves. Generally, The IR region begins after the visible region at 700 nm. The part of the spectrum between 2500 and 15000 nm is known as the infrared or **mid infrared**. The region between the 700 to 2,500 nm is called the **(Near IR) NIR**. The classical IR region is from 2,500 to 50,000 nm. The energies of IR radiation range from 48 kJ mol^{-1} at 2,500 nm to 2.4 kJ mol^{-1} at 50,000 nm.

The **near- and mid-IR regions** of the spectrum are most useful for quantitative and qualitative analysis of foods. Electron transitions cannot be due to these low energies, only vibrational changes within molecules occur.

Molecular Vibrations

The origin of the infrared spectra of molecules is the absorption of radiation at a specific wavelength by bonds in compounds, e.g. C-H, O-H, N-H and, to a lesser extent, S-H, as a result of **molecular vibrations**, which may include the stretching and contracting of bonds, the

changing of bond angles through bending and twisting, and various rocking motions. At exactly the correct frequency (the fundamental frequency), transitions occur from the ground state to the first vibrational excited state. Since vibrations can only occur at fixed frequencies, radiation is absorbed in discrete packets or quanta, and a molecule can only have characteristic absorption bands corresponding to these fixed frequencies. The amount of radiation absorbed is proportional to the number of similar bonds vibrating.

Note that the vibrational energy, and therefore the frequency of vibration, is directly proportional to the strength of the bond and inversely proportional to the mass of the molecular system. Thus, different chemical functional groups will vibrate at different frequencies. A vibrating molecular functional group can absorb radiant energy to move from the lowest ($\nu = 0$) vibrational state to the first excited ($\nu = 1$) state, and the frequency of radiation that will make this occur is identical to the initial frequency of vibration of the bond. This frequency is referred to as the fundamental absorption.

Mid -IR Spectroscopy

Mid-IR spectroscopy measures a sample's ability to absorb light in the 2.5-15 μm (2500 - 15000 nm) region. Fundamental absorptions are primarily observed in this spectral region. Mid-IR spectroscopy is very useful in the study of **organic compounds** because the absorption bands are related to the vibrational modes of specific **functional groups**. The positioning of the band and its intensity are correlated with the energy of the bond, its environment, and its concentration in the matrix, making mid-IR spectroscopy ideal for both qualitative and quantitative applications.

Mid infrared instruments are often used for identification purposes, such as in the study of plastics used for food packaging. They are also used for the estimation of food components where the routine analysis of a large number of samples is required. One particularly important application of infrared analysis is in the routine analysis of milk; the instruments used allow the measurement of all the major milk constituents, with the following specific wavelengths being used for each constituent:

3480 nm for fat (CH_2) groups

5723 nm for fat ($\text{C}=\text{O}$) groups

6465 nm for protein (N-H) peptide groups

9610 nm for lactose (C-OH) groups

4300 nm for water (H-O-H) groups

Infrared spectrophotometers obey the same fundamental principles as those involving UV/visible radiation, but use different materials in their construction. Typical sources of radiation include **Nichrome coils** raised to incandescence by resistive heating, whilst detection may be achieved by the use of **thermal sensitive devices**. Cells used in infrared studies are generally manufactured from metal halide salts since these provide the required transmittance of infrared beams. As with UV/visible spectrophotometry, the radiation is passed through a series of mirrors and split into sample and reference beams. One beam is passed through the sample and one through the reference solution. The wavelength, as indicated above, is chosen for the specific component being estimated, e.g. 6465 nm for protein, and the absorbance is measured. This is then converted to a digital readout after subtracting the difference between the sample and the reference.

In the compositional analysis of food products by infrared analysis, **calibration** of the instruments is required. In **infrared milk analysis (IRMA)**, for example, instruments are calibrated for lactose, protein and fat against standard methods of analysis.

For general food analysis, major use has been made in recent times of near infrared (NIR) using wavelengths in the region of 800 nm to 2500 nm. Although absorptivity of NIR is 10-1000 times less than mid infrared bands, NIR beams penetrate deeper into the food sample, giving a more representative analysis. NIR analyses are based on the fact that certain combinations of fundamental vibrations can also absorb radiation giving rise to a number of additional possible absorption bands. At approximately **double frequency**, transition occurs to the second level (the first overtone), and for approximately the triple frequency transition to the third level may occur (the second overtone), and so on. With NIR, since not all constituents absorb in this region of the spectrum, the region of overtones and combinations is less complex, and the constituents are thus more readily detected than with mid infrared.

Tungsten filament lamps may be used as a source of radiation for NIR instruments but, unlike mid infrared instruments, detection requires photoelectric devices rather than thermal sensitive ones.

NIR makes use of sophisticated statistical techniques to correlate absorbance, or transmittance, measurements with the chemical composition of the sample. Its **advantages** lie in the speed of analysis possible and the relative ease of sample handling. Its major **disadvantages** include the high initial cost of the instruments and in the requirement for a large number of samples for calibration. The technique finds particular use in the milling industry for the routine measurement of wheat hardness, and may also be used to estimate the quality of tea and coffee.



Fig. 7.9 IR spectrophotometer

Atomic Spectrometry

Atomic spectroscopy (AS) measures the concentration of **chemical elements in a sample**. When elements are transformed into atomic vapor at high temperatures, emission or absorption of light may occur and this can be accurately measured at a unique resonant wavelength. Modern AS can be divided into three related techniques. Whereas **atomic absorption spectrometry (AAS)** measures the quantity of light absorbed by atoms of the analyte, **atomic emission spectrometry (AES)** and **atomic fluorescence spectrometry (AFS)** measure the quantity of radiation emitted by analyte atoms. The application of atomic spectroscopic techniques for the analysis of **major, minor, and trace elements in foods**.

As the names imply, atomic absorption spectroscopy (AAS) quantifies the **absorption** of electromagnetic radiation by well-separated neutral atoms, while atomic emission spectroscopy (AES) measures **emission** of radiation from atoms in excited states.

AAS and AES allow accurate measurements of mineral elements even in the presence of other components because the atomic absorption and emission spectra are unique for each individual element. These instrumental methods have largely replaced traditional wet chemistry methods for food mineral analysis, although traditional methods for calcium, chloride, fluoride, and phosphorus remain in use today.

General Principles

Energy Transitions in Atoms

Atomic absorption spectra are produced when **ground-state** atoms absorb **energy** from a radiation source. Atomic emission spectra are produced when neutral atoms in an **excited state** emit energy on returning to the ground state or a lower-energy state.

Each element has a **unique** set of allowed electronic transitions and therefore a unique spectrum, enabling accurate identification and quantification even in the presence of other elements.

The absorption and emission spectra of **sodium** are shown in **Figure 7.10**. For absorption, transitions involve primarily the excitation of electrons in the ground state, so the number of transitions is relatively small. Emission, on the other hand, occurs when electrons in various excited states fall to lower energy levels including, but not limited to, the ground state. Therefore, the emission spectrum has more lines than the absorption spectrum of the same element as illustrated in Figure 7.10.

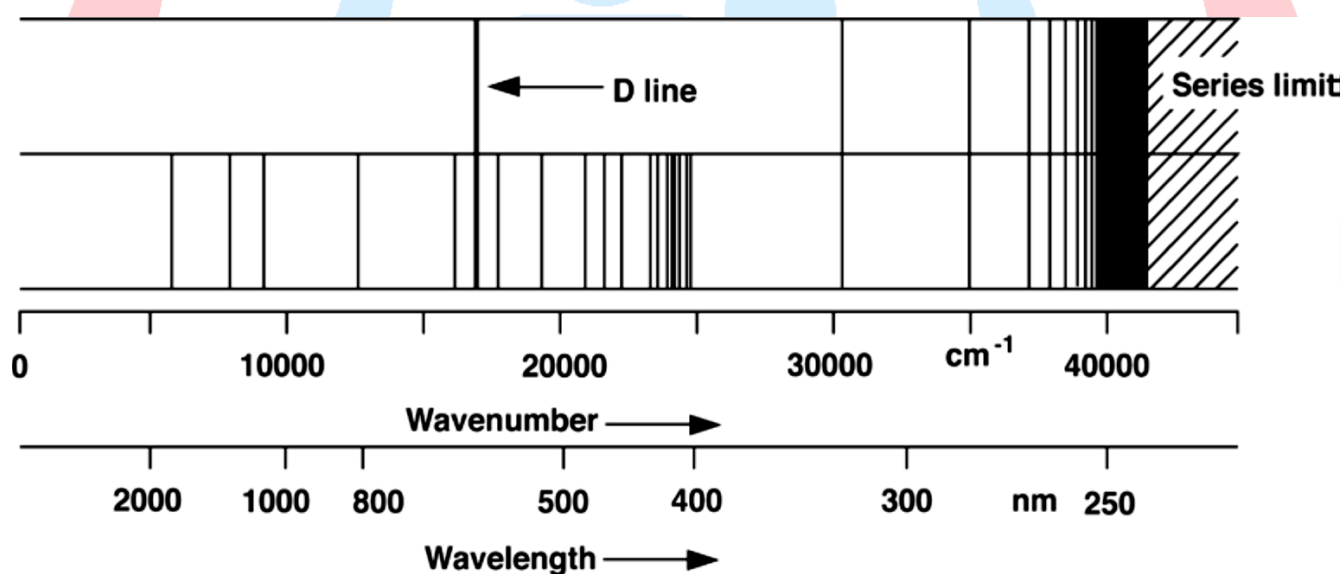


Fig. 7.10 Spectra for sodium. The upper spectrum (a) is the absorption spectrum, and the lower (b) is the emission spectrum

Atomization

Atomic spectroscopy requires that atoms of the element of interest to be in the atomic state (not combined with other elements in a compound) and to be well separated in space. In foods, virtually all elements are present as compounds or complexes and therefore must be **converted** to neutral atoms (i.e., atomization) before atomic absorption or emission measurements can be made. Atomization is usually accomplished by exposing a solution containing the analyte

(the substance being measured) as a fine mist to **high temperatures**, typically in a flame or plasma. The solvent quickly **evaporates**, leaving solid particles of the analyte that **vaporize and decompose to atoms** that may absorb radiation (atomic absorption) or become excited and subsequently emit radiation (atomic emission). This process is shown schematically in **Figure 7.11**.

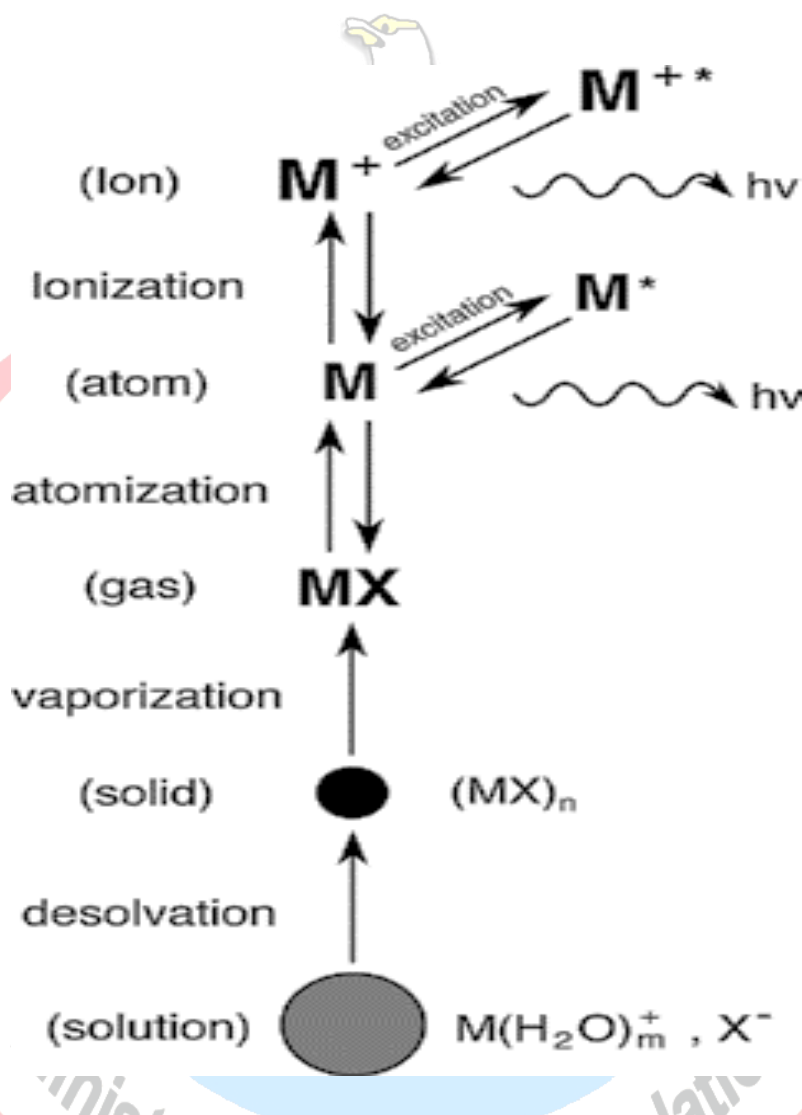


Fig. 7.11 Atomization of an element in a flame or plasma

Atomic Absorption Spectroscopy (AAS)

AAS is a relatively simple method and was the most widely used form of atomic spectroscopy in food analysis for many years. The process of atomic absorption is illustrated in **Figure 7.12**.



Fig. 7.12 Atomic Absorption Process

Simple statements can express the **basic principles of atomic absorption spectroscopy**:

- All atoms can absorb light.
- The wavelength at which light is absorbed is **specific** for each element. If a sample containing nickel, for example, together with elements such as lead and copper is exposed to light at the characteristic wavelength for nickel, then only the nickel atoms will absorb this light.
- The amount of light absorbed at this wavelength will increase as the number of atoms of the selected element in the light path increases, and is proportional to the concentration of absorbing atoms.
- The relationship between the amount of light absorbed and the concentration of the analyte present in known standards can be used to determine unknown concentrations by measuring the amount of light they absorb. An atomic absorption spectrometer is simply an instrument in which these basic principles are applied to practical quantitative analysis.
- Two types of atomization are commonly used in AAS: **flame atomization** and **electrothermal (graphite furnace) atomization**.

Principles of Flame Atomic Absorption Spectroscopy

A schematic diagram of a flame atomic absorption spectrometer is shown in **Figure 7.13**. In flame AAS, a **nebulizer-burner system** is used to convert a sample solution into an atomic vapor. It is important to note that the sample must be in solution (usually an aqueous solution) before it can be analyzed by flame AAS. The sample solution is **nebulized** (dispersed into tiny droplets), mixed with fuel and an oxidant, and burned in a flame produced by oxidation of the fuel. Atoms and ions are formed within the hottest portion of the flame as the sample solution goes through the process of desolvation, vaporization, atomization, and ionization (**review Fig. 7.11**). Atoms and ions of the same element absorb radiation of different wavelengths and produce unique spectra. Therefore, it is desirable to choose a flame temperature that will maximize atomization and minimize ionization because atomic absorption spectrometers are tuned to measure **atomic absorption, not ionic absorption**.

Once the sample is atomized in the flame, the quantity of the analyte element is measured by determining the attenuation (decrease in intensity) of a beam of radiation passing through the flame, due to atomic absorption of incident radiation by the analyte element.

For the measurement to be specific for the analyte element (**Figure 7.14**), the radiation source ideally should **emit radiation** of the exact discrete wavelengths that **only** the analyte element is capable of **absorbing**. This can be accomplished by using **lamps with cathodes fabricated**

with the element to be determined. Thus, the beam of radiation emitted from the lamp is the emission spectrum of the element. The spectral line of interest is isolated by passing the beam through a **monochromator** so that only radiation of a very narrow band width reaches the **detector**.

Note also that the line width of the radiation from the source is narrower than the corresponding line width in the absorption spectrum. This is because the higher temperature of the flame causes a broadening of the line width.

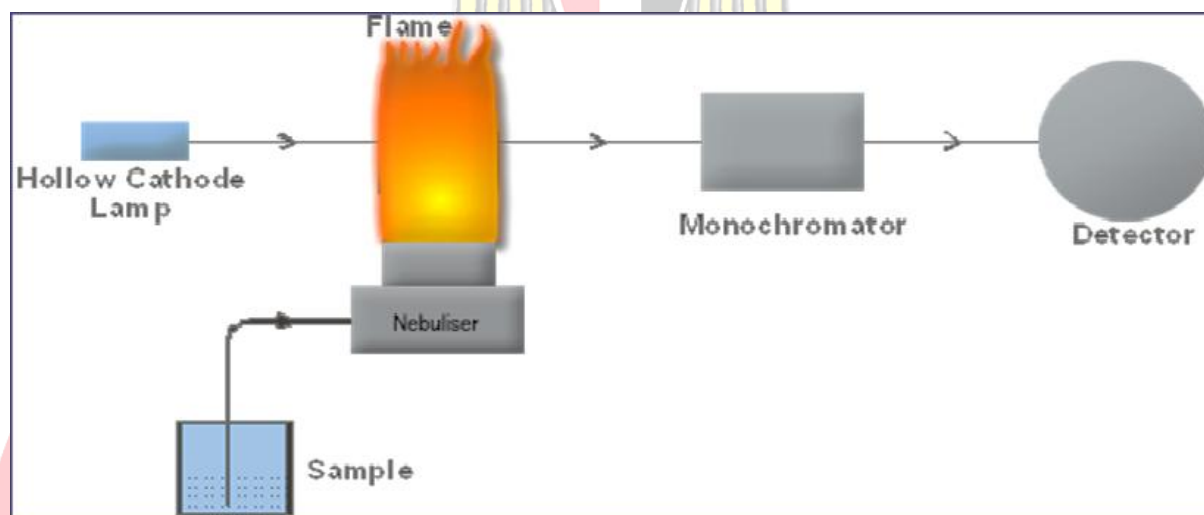


Fig. 7.13 A schematic diagram of a flame atomic absorption spectrometer

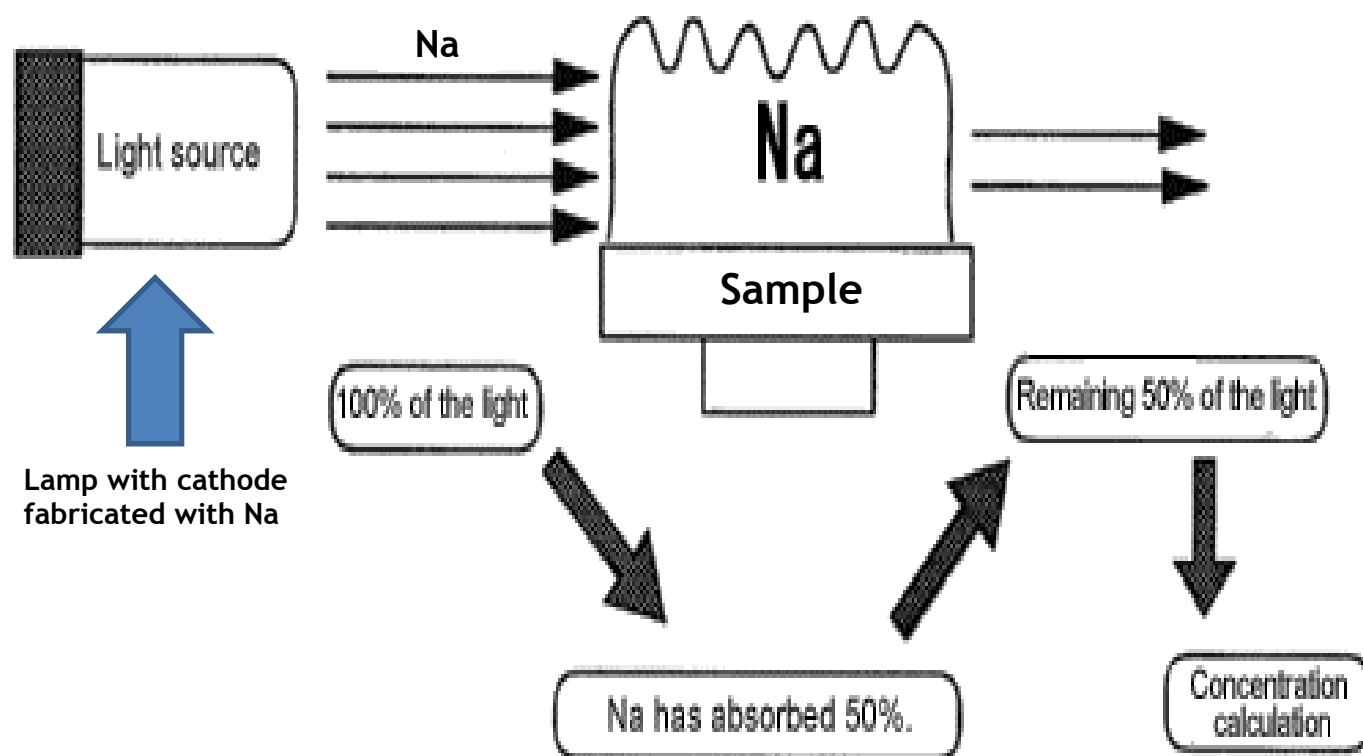


Fig. 7.14 Attenuation of a beam of radiation as it passes through the Na atoms in flame

The amount of radiation absorbed by the analyte element is given by **Beer's law**:

$$A = \text{Log} (I_0 / I) = \epsilon LC \quad \text{Eq. 7.8}$$

Where:

A = absorbance

I_0 = intensity of radiation incident on the flame

I = intensity of radiation exiting the flame

ϵ = molar absorptivity

L = path length through the flame

C = concentration of atoms in the flame



Principles of Electrothermal (Graphite Furnace) Atomic Absorption Spectroscopy

Electrothermal AAS is identical to flame AAS except for the atomization process. In electrothermal AAS, the sample is heated **electrically** in stages inside a **graphite tube**, commonly known as **graphite furnace**, to achieve **atomization**. The tube is aligned to the path of the radiation beam to be absorbed by the atomized sample and absorbance is determined. Electrothermal AAS requires smaller sample sizes and offers lower detection limits. Disadvantages are the added expense of the graphite furnace, lower sample throughput, higher matrix interferences, and lower precision.

Atomic Absorption Instrumentation

There are **five basic components** of an atomic absorption instrument:

1. **The light source** that emits the spectrum of the element of interest
2. **Atomizer**, which atoms of the sample are produced, usually a nebulizer-burner system or a graphite furnace
3. **A monochromator** for light dispersion, usually an UV-Vis grating monochromator
4. **A detector**, which measures the light intensity and amplifies the signal, a photomultiplier tube (PMT) or a solid-state detector (SSD).
5. **A display** that shows the reading after it has been processed by the instrument electronics, an analog or a digital readout.

There are two basic types of atomic absorption instruments: single-beam and double-beam.

Single-Beam Atomic Absorption Spectrometer

A schematic diagram of a single-beam atomic absorption instrument is shown in Figure 7.15.

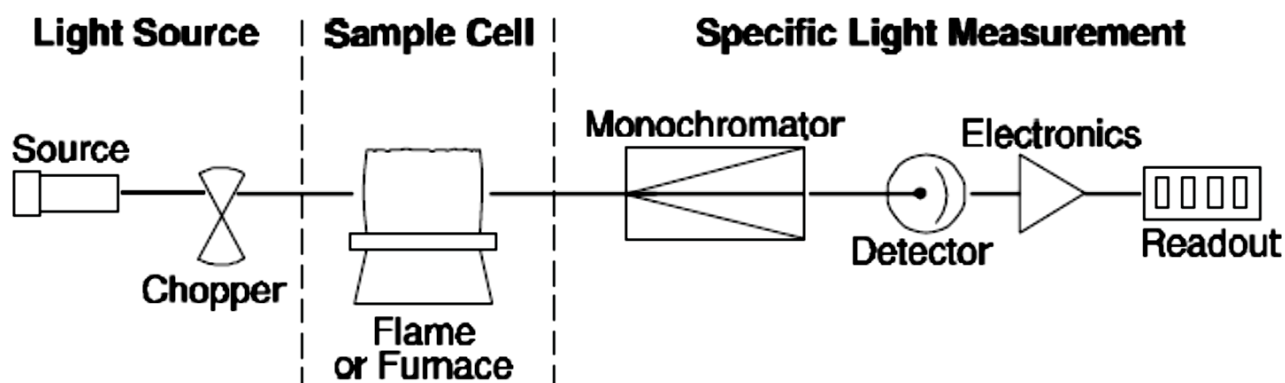


Fig. 7.15 Single-Beam Atomic Absorption Spectrometer

The light source (hollow cathode lamp or electrodeless discharge lamp) emits a spectrum specific to the element of which it is made, which is focused through the sample cell into the **monochromator**. The light source must be electronically modulated or mechanically **chopped** to differentiate between the light from the source and the emission from the sample cell. The monochromator disperses the light and the specific wavelength of light isolated passes to the **detector**, which is usually a photomultiplier tube. An electrical current is produced depending on the light intensity and processed by the instrument electronics. The electronics will measure the amount of light attenuation in the sample cell and convert those readings to the actual sample concentration. With single-beam systems, a short warm-up period is required to allow the source lamp to stabilize.

Double-Beam Atomic Absorption Spectrometer

A schematic diagram of a double-beam system is shown in Figure 7.16. The light from the source lamp is divided into a sample beam, which is focused through the sample cell, and a reference beam, which is directed around the sample cell. In a double-beam system, the readout represents the ratio of the sample and reference beams. Therefore, fluctuations in source intensity do not become fluctuations in instrument readout, and stability is enhanced. Generally, analyses can be performed immediately with no lamp warm-up required.

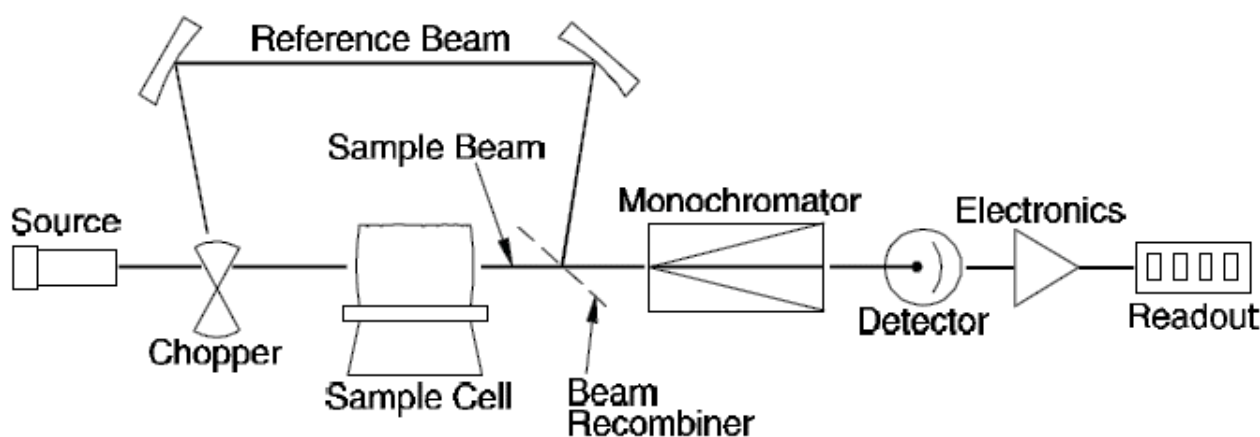


Fig. 7.16 Double-Beam Atomic Absorption Spectrometer

Light Sources for Atomic Absorption

Since atoms absorb light at very specific wavelengths, it is necessary to use a narrow-line source which emits the narrow-line spectra of the element of interest. Narrow-line sources provide high intensity and make atomic absorption a specific analytical technique. The main sources used for atomic absorption are the **hollow cathode lamp (HCL)** and the **electrode-less discharge lamp (EDL)**.

A **hollow cathode lamp (HCL)** consists of a hollow tube filled with argon or neon gas, an anode made of tungsten, and a cathode made of the metallic form of the element being measured (**Figure 7.17**). When voltage is applied across the electrodes, the lamp emits radiation characteristic of the metal in the cathode (**Figure 7.18**). For example, if the **cathode is made of iron, an iron spectrum will be emitted**. As the radiation passes through the flame containing the atomized sample, only iron atoms (not atoms of other elements) will absorb this radiation because the emitted wavelengths from the HCL are **specific** for iron atoms. Of course, this means that it is necessary to use a different lamp for each element analyzed. The hollow cathode lamp is an excellent, bright, stable line source for most elements.

An **electrode-less discharge lamp (EDL)** contains no electrodes but a hollow glass vessel filled with an inert gas plus the element of interest. The discharge is produced by a **high-frequency generator coil** rather than an electric current. EDLs are suitable for volatile elements such as arsenic, mercury, and cadmium.

Multi-element Lamps, which the cathode of a hollow cathode lamp is generally constructed from a very pure metal resulting in a very pure emission spectrum. It is, however, possible to construct a cathode from a **mixture or alloy of several metals**. The resulting "multi-element" lamp can be used as a source for all the metals contained in the cathode. There is a wide variety of multielement lamp combinations available. Not all metals can be used in combination due to metallurgical properties or spectral limitations.

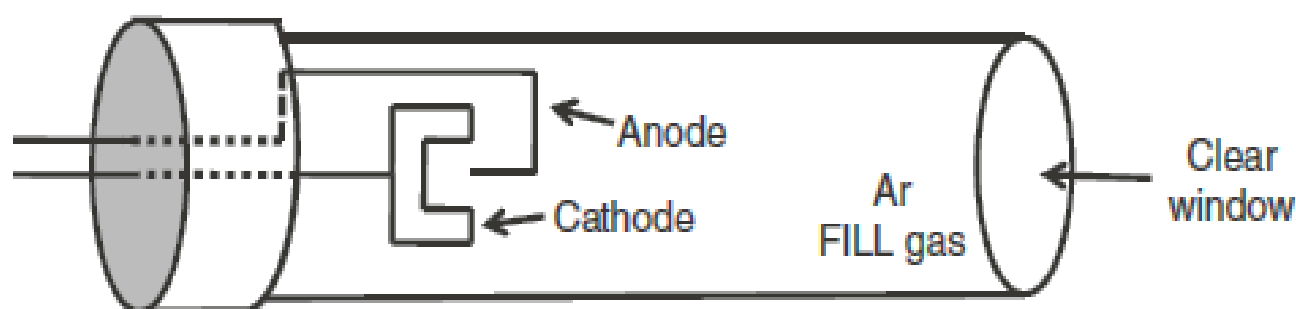


Fig. 7.17 Schematic representation of a hollow cathode lamp

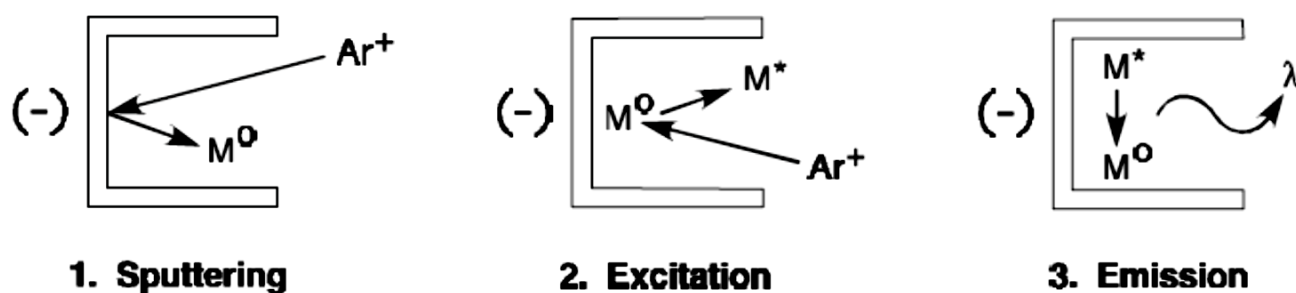


Fig. 7.18 Hollow Cathode Lamp Emission Process

Radiation reaching the monochromator comes from three sources:

- (1) Attenuated beam from the HCL (specific emission)
- (2) Emission from sample atoms (including both analyte and non-analyte atoms) that were excited by the flame (nonspecific emission)
- (3) Radiation resulting from the combustion of the fuel to create the flame (nonspecific emission).

Instruments are designed to eliminate nonspecific emissions from reaching the detector. This is accomplished by positioning a monochromator between the flame and the detector. The monochromator disperses wavelengths of light that are not specific to the analyte element and isolates a line that is specific. Thus, radiation reaching the detector is the sum of radiation from the attenuated HCL beam and radiation emitted by excited analyte atoms in the flame. Since we are interested only in the amount of HCL radiation absorbed by analyte atoms in the flame, it is necessary to correct for emission from excited analyte atoms in the flame. This is accomplished by positioning a **beam chopper** perpendicular to the light path between the HCL and the flame. A beam chopper is a disk with segments removed. The disk rotates at a constant speed so that the light emitted from the HCL reaching the detector is either unimpeded or blocked at regular intervals, i.e., it is alternating. In contrast, emission from excited analyte atoms in the flame reaching the detector is continuous. The instrument electronics subtracts the continuous emission signal from the alternating emission signal so only the signal from the attenuated HCL beam is recorded in the readout.

Atomizers for Atomic Absorption

Flame and **graphite furnace** atomizers are the two common types of atomizers used in AAS. The **flame atomizer** consists of a **nebulizer** and a **burner**. The nebulizer is designed to convert the sample solution into a fine mist or aerosol. This is accomplished by aspirating the sample through a capillary into a chamber through which oxidant and fuel are flowing.

Flame characteristics may be manipulated by adjusting oxidant/fuel ratios and by choice of oxidant and fuel. Air-acetylene and nitrous oxide-acetylene are the most commonly used oxidant-fuel mixtures.

Flame atomizers have the advantage of being stable and easy to use. However, sensitivity is relatively low because much of the sample never reaches the flame and the residence time of the sample in the flame is short.

The **graphite furnace** is typically a cylindrical graphite tube connected to an electrical power supply. The sample is injected into the tube through an inlet using a microliter syringe with sample volumes ranging from 0.5 to 100 μL . During operation, the system is flushed with an inert gas to prevent the tube from burning and to exclude air from the sample compartment. The tube is heated electrically in stages: first, the sample solvent is evaporated, then the sample is ashed, and finally the temperature is rapidly increased to $\sim 1700 - 2700^\circ \text{C}$ to quickly vaporize and atomize the sample.

General Procedure for Atomic Absorption Analysis

The basic design of all atomic absorption spectrometers is similar, operation procedures do vary from one instrument to another. Method of analysis provide pertinent information for the analysis of each particular element (wavelength and slit width requirements, interferences and corrections, flame characteristics).

Safety Precautions

General laboratory safety protocols and procedures as well as safety precautions recommended by the instrument manufacturers must be followed carefully to avoid personal injuries or costly accidents. The most commonly used fuel sources in flame AAS are mixtures of air-acetylene and nitrous oxide-acetylene. **Acetylene is an explosive gas.** Proper ventilation must be in place before operation. The exhaust vent should be positioned directly above the burner to avoid the buildup of unburned fuel or any potentially hazardous toxic fumes. Flame atomic absorption spectrometers should never be left unattended while in operation.

Calibration

Quantitative measurements in atomic absorption are based on Beer's Law, which states that concentration is proportional to absorbance.

As illustrated in Figure 7.19, a plot of absorbance versus concentration will deviate from linearity predicted by Beer's law when the concentration exceeds a certain level. Therefore, properly constructed calibration curves using pure standards are essential for accurate quantitative measurements., the linear range of an element should be established by running a series of standards of increasing concentration and plotting absorbance versus concentration. The concentration of the unknown sample solution should be adjusted so that the measured absorbance always falls within the linear range of the calibration curve.

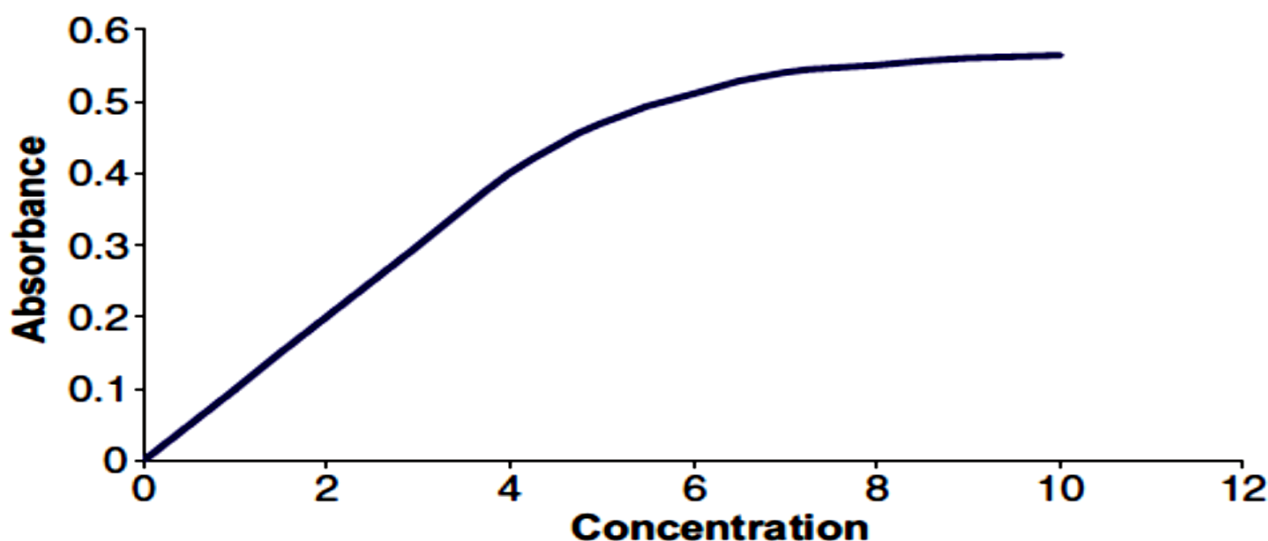


Fig. 7.19 A plot of absorbance vs. concentration showing nonlinearity above a certain concentration

Atomic Emission Spectroscopy (AES)

In contrast to AAS, the source of the **measured radiation in AES is the excited atoms in the sample**, not radiation from a HCL. **Figure 7.20** shows a simplified diagram of an atomic emission spectrometer. Sufficient energy is first applied to the sample to excite atoms to higher-energy levels; emissions of wavelengths characteristic of individual elements are then measured when electrons from excited atoms move back to the ground state or a lower-energy state. Since each element has a unique set of energy levels, each element also has a unique set of wavelengths at which it will emit energy. Thus, the wavelengths of light emitted by the atoms or ions are specific to the elements which are present in the sample.

It is also possible to determine the concentration of analyte that is present in a sample by measuring the amount of light emitted and comparing this value with the amount of light emitted by known standards.

Energy for excitation may be produced by **heat** (usually from a flame), **light** (from a laser), **electricity**, or **radio waves** (Inductively coupled plasma ICP).

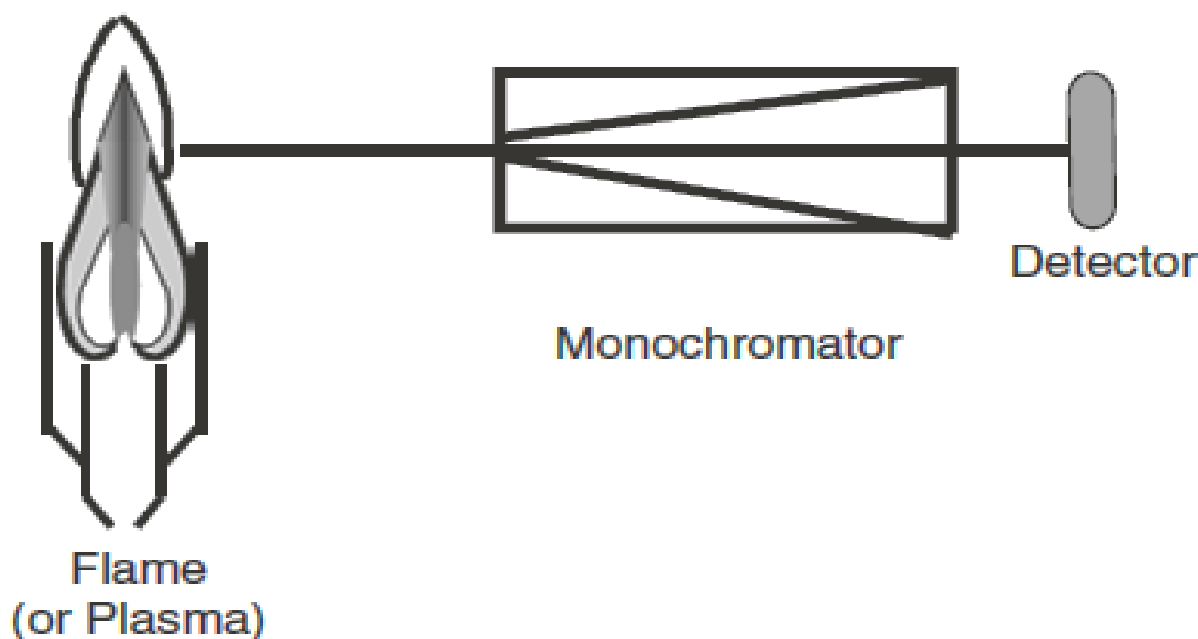


Fig. 7.20 A simplified diagram of an atomic emission spectrometer

The two most common forms of AES used in food analysis are **flame emission spectroscopy** and **inductively coupled plasma-optical emission spectroscopy (ICP-OES)**.

Principles of Flame Emission Spectroscopy

Flame emission spectrometers employ a nebulizer burner system to atomize and excite the atoms of the elements being measured. The flame with the excited atoms serves as the radiation source, so an external source (the HCL with the beam chopper) is **not required**. Otherwise, instrumentation for flame emission spectroscopy is essentially identical to that for AAS. Many modern atomic absorption spectrometers can also be operated as flame emission spectrometers.

Principles of Inductively Coupled Plasma-Optical Emission Spectroscopy

ICP-OES differs from flame emission spectroscopy in that it uses an argon plasma as the excitation source. A **plasma** is defined as a gaseous mixture containing significant concentrations of cations and electrons. Temperatures in argon plasmas could be as high as $10,000^{\circ}\text{K}$, with analyte excitation temperature typically ranging from $6,000$ to $7,000^{\circ}\text{K}$.

The extremely high temperatures and the inert atmosphere of argon plasmas are ideal for the atomization, ionization, and excitation of the analyte atoms in the sample. The low oxygen content reduces the formation of oxides, which is sometimes a problem with flame methods. The relatively uniform temperatures in plasmas (compared to non-uniform temperatures in flames) give good linear responses over a wide concentration range.

Instrumentation for Inductively Coupled Plasma-Optical Emission Spectroscopy

Inductively coupled plasma-optical emission spectrometers typically consist of the following components (**Figure 7.21**):

1. Argon plasma torch
2. Monochromator, polychromator, or echelle optical system
3. Detector(s), a single or multiple PMT(s) or solid-state array detector(s)
4. Computer for data collection and treatment

Argon Plasma Torch

The plasma torch (**Figure 7.22**) consists of three concentric quartz tubes centered in a copper coil, called the **load coil**. During operation of the torch, a stream of argon gas flows through the outer tube, and **radio frequency (RF)** power is applied to the load coil, creating a **magnetic field oscillating** at the frequency of the RF generator (usually 27 MHz or 40 MHz).

The plasma is started by **ionizing argon atoms** with an **electric spark** to form **argon ions and electrons**. The oscillating magnetic field couples with the argon ions and electrons, forcing them to flow in an annular (ring-shaped) path. Heating does not involve burning fuel to directly heat and atomize the sample, as is the case with flame AAS (**argon is a noble gas and will not**

combust). Rather, heating is accomplished by **transferring RF energy to free electrons and argon ions** in a manner similar to the transfer of microwave energy to water in a microwave oven. These **high-energy electrons** in turn collide with argon atoms, generating even more electrons and argon ions and causing a **rapid increase in temperature** to approximately 10,000 °K. The process continues until about 1 % of the argon atoms are ionized. At this point, the plasma is very stable and self-sustaining for as long as the RF field is applied at constant power. The transfer of energy to a system through the use of electromotive forces generated by magnetic fields is known as **inductive coupling**, hence the name ICP.

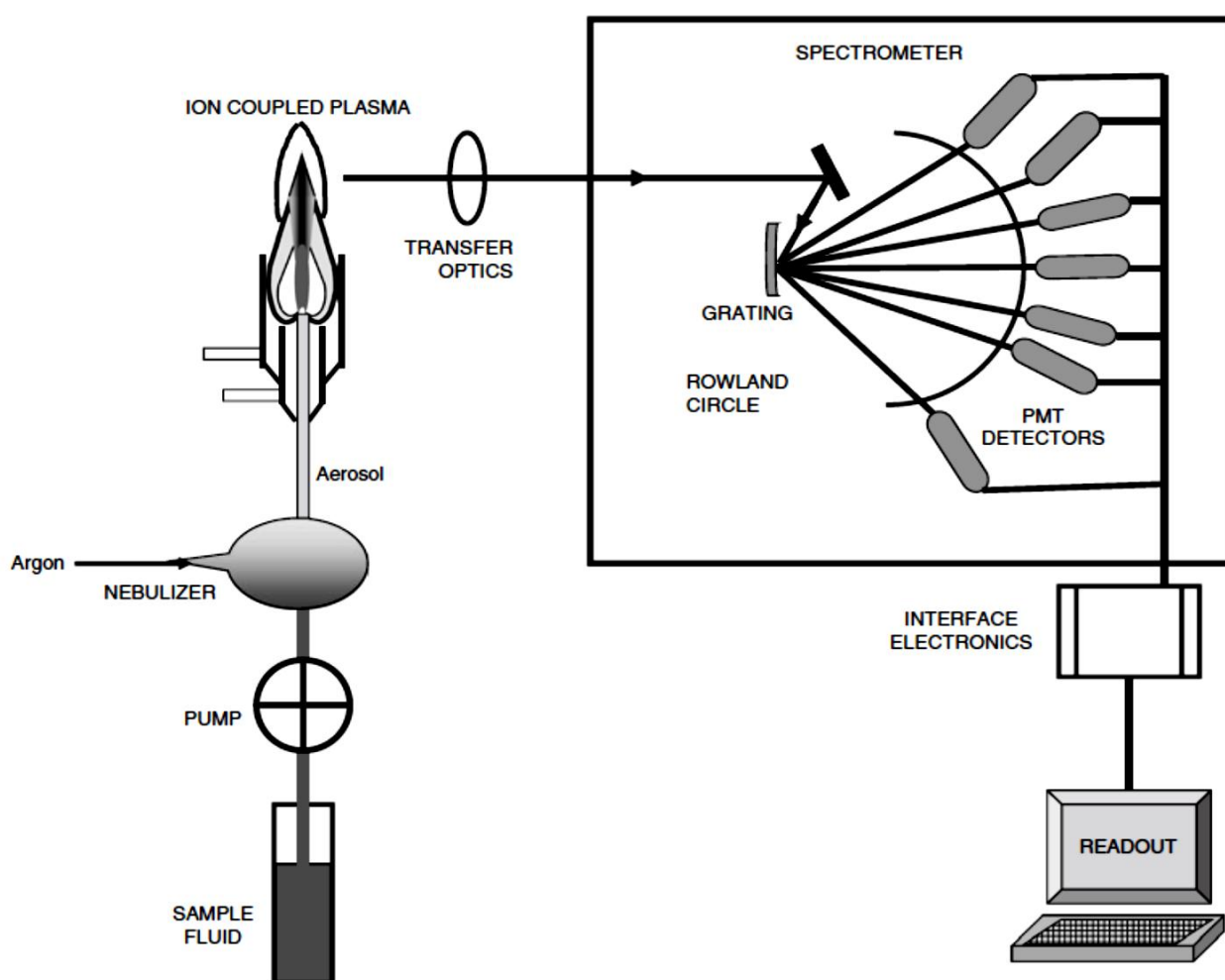


Fig. 7.21 Schematic of an inductively coupled plasma-optical emission simultaneous spectrometer

Sample Introduction and Analyte Excitation

Samples are nebulized and introduced as aerosols carried by another stream of argon gas in the inner tube inside the annulus of the plasma at the base of the RF load coil. The sample goes through the process of desolvation, vaporization, atomization, ionization, and excitation as shown in **Figure 7.11**, when electrons are accelerated in a magnetic field, they acquire enough kinetic energy to excite analyte atoms and ions upon collision.

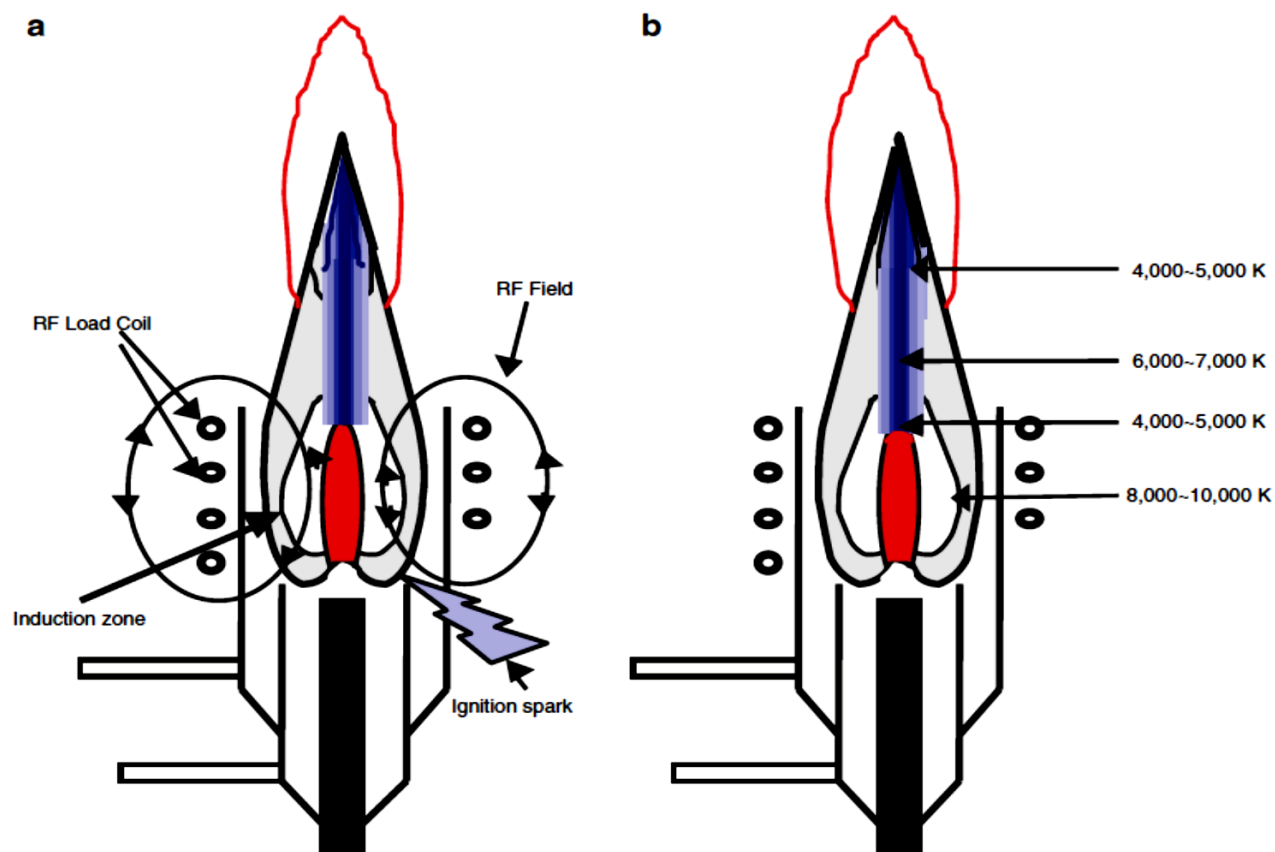


Fig. 7.22 The ICP plasma: (a) the process by which the plasma is formed and sustained and (b) the temperature distribution of the plasma

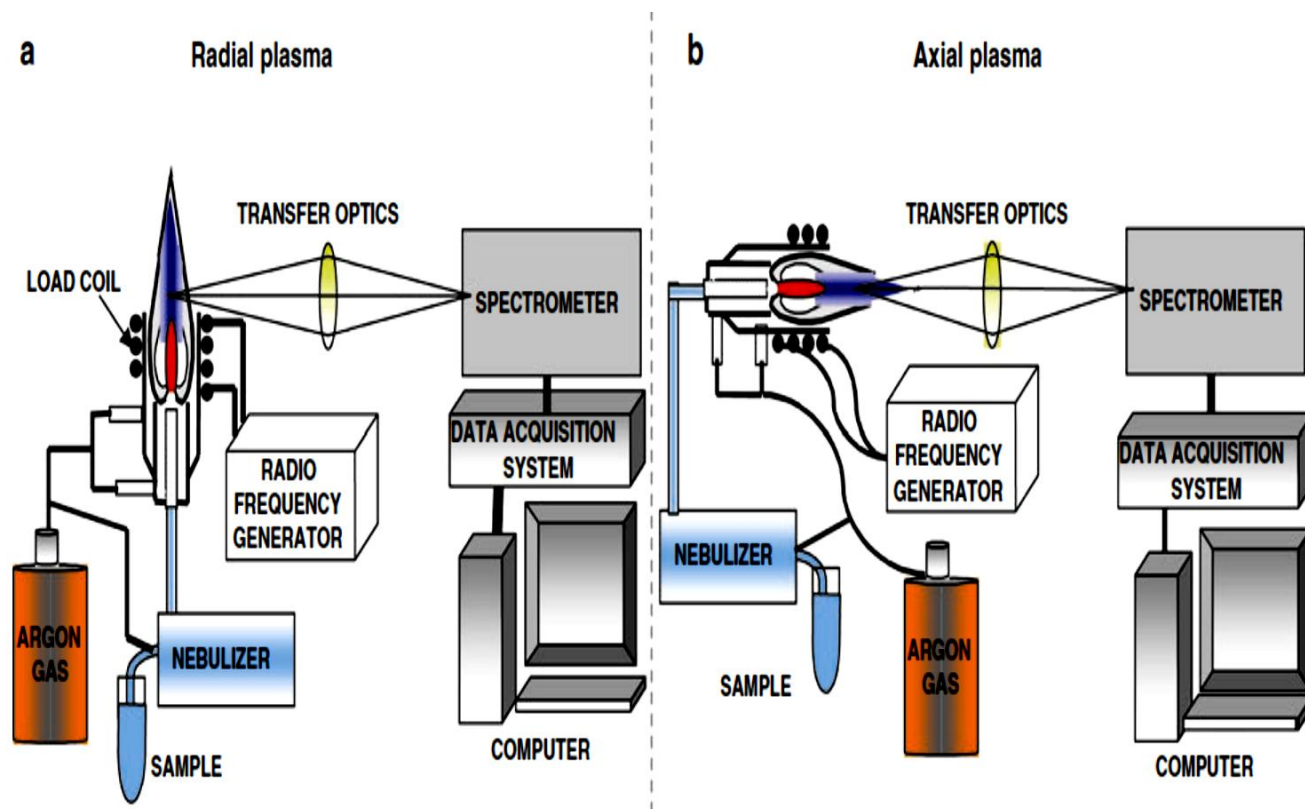


Fig. 7.23 Major components and typical layout of (a) a radially viewed ICP-OES instrument and (b) an axially viewed ICP-OES instrument.

Mass Spectroscopy

Mass spectrometry is a powerful analytical technique used to quantify known materials, to **identify unknown compounds** within a sample, and to **elucidate the structure and chemical properties of different molecules**. The complete process involves the conversion of the sample into gaseous ions, with or without fragmentation, which are then characterized by their **mass to charge ratios (m/z)** and **relative abundances**. Important development led to the rapid rise in popularity of MS as an analytical technique. It was the development of hyphenated MS techniques, which coupled the separation techniques of gas chromatography (GC) or liquid chromatography (LC) (**Chap. 8**) to MS. This coupling of chromatography and MS dramatically lowered the detection limits for quantitative analysis while simultaneously increasing the confidence of measurement through high specificity.

This technique basically studies the effect of ionizing energy on molecules. It depends upon chemical reactions in the gas phase in which sample molecules are consumed during the formation of ionic and neutral species.

Basic Principle

The power of the MS technique is due to its ability to place a charge on a molecule, thereby converting it to an ion in a process called **ionization**. The generated ions are then separated according to their **mass-to-charge ratio (m/z)** by subjecting them to a combination of radio-frequency (**RF**) and electrostatic fields in a **mass analyzer** and finally detected by highly sensitive **detectors**. The resulting signals from the detectors are digitized and processed by software to display the information as a mass spectrum, which reveals its molecular mass and its structural composition, leading to identification.

Components

The instrument consists of three major components (**Figure 7.24**):

1. **Ion Source:** For producing gaseous ions from the substance being studied.
2. **Analyzer:** For resolving the ions into their characteristics mass components according to their mass-to-charge ratio.
3. **Detector System:** For detecting the ions and recording the relative abundance of each of the resolved ionic species.

In addition, a sample introduction system is necessary to admit the samples to be studied to the ion source while maintaining the high vacuum requirements ($\sim 10^{-6}$ to 10^{-8} mm of mercury) of the technique; and a computer is required to control the instrument, acquire and manipulate data, and compare spectra to reference libraries.

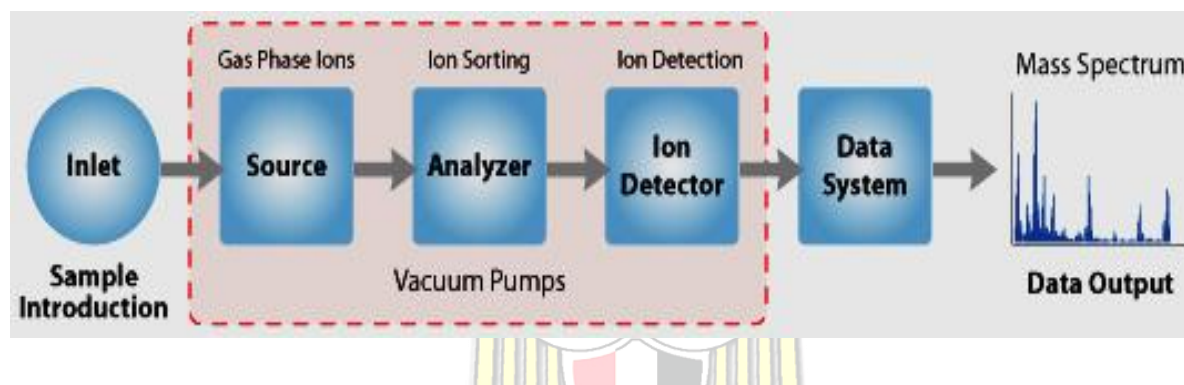


Fig. 7.24 Components of a Mass Spectrometer

Figure 7.24 is a block diagram that shows the basic parts of a mass spectrometer. The inlet transfers the sample into the vacuum of the mass spectrometer. In the source region, neutral sample molecules are ionized and then accelerated into the mass analyzer. The mass analyzer is the heart of the mass spectrometer. This section separates ions, either in space or in time, according to their mass to charge ratio. After the ions are separated, they are detected and the signal is transferred to a data system for analysis. All mass spectrometers also have a vacuum system to maintain the low pressure, which is also called high vacuum, required for operation. High vacuum minimizes ion-molecule reactions, scattering, and neutralization of the ions.

With all the above components, a mass spectrometer should always **perform the following processes:**

1. Produce ions from the sample in the ionization source.
2. Separate these ions according to their mass-to-charge ratio in the mass analyzer.
3. Eventually, fragment the selected ions and analyze the fragments in a second analyzer.
4. Detect the ions emerging from the last analyzer and measure their abundance with the detector that converts the ions into electrical signals.
5. Process the signals from the detector that are transmitted to the computer and control the instrument using feedback.

Chapter 8

Chromatography Principles and Applications in Food Analysis

Objectives

- After reading this chapter, you should be able to:
 - Recognize chromatography instruments principles and applications in food analysis.

Introduction

The term chromatography is derived from the Greek *chroma* meaning colour, and originates from the original use of chromatography for the separation of plant leaf pigments as coloured bands on a column of calcium carbonate (**Figure 8.1**). The principle has developed today into a number of general types and involving a number of different techniques.

“Chromatography is an analytical technique wherein a sample mixture under test is separated into different components.”

Chromatography principle:

When a **solute** in a solvent (or a **mobile phase**) is passed through or around the outside of a matrix (or a **stationary phase**), interactions occur between the solute and the stationary phase. Solutes with different properties are separated based on differences in these interactions.

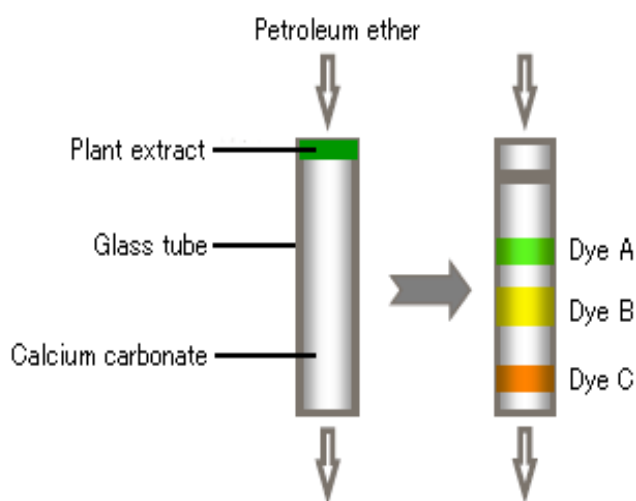


Fig. 8.1 Separation of plant leaf pigments

The basic principles of chromatography involve the interaction between three components:

- **Stationary phase:** This phase is always composed of a “solid” phase or “a layer of a liquid adsorbed on the surface a solid support”.
- **Mobile phase:** This phase is always composed of “liquid” or a “gaseous component.”
- **Separated molecules (solute).**

• The type of interaction between stationary phase, mobile phase, and substances contained in the mixture is the basic component effective on separation of molecules from each other. Chromatography methods based on partition are very effective on separation, and identification of small molecules as amino acids, carbohydrates, and fatty acids. However, affinity chromatography and ion-exchange chromatography are more effective in the separation of macromolecules as nucleic acids, and proteins.

Stationary phase (SP) in chromatography is a solid phase or a liquid phase coated on the surface of a solid phase. **Mobile phase (MP)** flowing over the stationary phase is a gaseous or liquid phase. If mobile phase is liquid it is termed as **liquid chromatography (LC)**, and if it is gas then it is called **gas chromatography (GC)**. Gas chromatography is applied for gases, and mixtures of volatile liquids, and solid material. Liquid chromatography is used especially for thermal unstable, and non-volatile samples.

The purpose of applying chromatography which is used as a method of **quantitative analysis** apart from its separation, is to achieve a satisfactory separation within a suitable time interval. Various chromatography methods have been developed to that end. Some of them include column chromatography, thin-layer chromatography (TLC), paper chromatography, gas chromatography, ion exchange chromatography, gel permeation chromatography, high-pressure liquid chromatography, and affinity chromatography.

General Terminology

Chromatography is a general term applied to a wide variety of separation techniques based on the partitioning or distribution of a sample (**solute**) between a moving or mobile phase and a fixed or stationary phase.

Partition (K) or distribution (D) coefficient is the ratio of concentration of solute in stationary phase to concentration of solute in mobile phase.

$$K = \frac{\text{Concentration of solute in phase 1}}{\text{Concentration of solute in phase 2}} \quad \text{Eq. 8.1}$$

Table 8.1 summarizes some of the chromatographic procedures or methods that have been developed on the basis of different mobile-stationary phase combinations. Inasmuch as the nature of interactions between solute molecules and the mobile or stationary phases differ, these methods have the ability to separate different kinds of molecules.

Table 8.1 Characteristics of different chromatographic methods

Method	Mobile/phase	Stationary phase	Separation Mechanism
Gas-liquid chromatography	Gas	Liquid	Molecular size/polarity
Gas-solid chromatography	Gas	Solid	Molecular size/polarity
Reversed-phase chromatography	Polar liquid	Nonpolar liquid or solid	Molecular size/polarity
Normal-phase chromatography	Less polar liquid	More polar liquid or solid	Molecular size/polarity
Ion-exchange chromatography	Polar liquid-Ionic solid	Ionic solid	Molecular charge
Size-exclusion chromatography	Liquid	Solid	Molecular size
Affinity chromatography	Water	Binding sites	Specific structure

Three major types of support are used in chromatography, **paper, thin-layer and column**, giving rise to the following general categories of chromatographic methods available to the food analyst.

A. Planar chromatography

1. Paper chromatography
2. Thin-layer chromatography (TLC)

B. Column chromatography

1. Gas chromatography (GC)
2. Liquid chromatography (LC)
 - (a) Liquid-liquid chromatography (LLC)
 - (b) Liquid-solid chromatography (LSC)

What differences between adsorption chromatography & partition chromatography?

Adsorption (Liquid-Solid) Chromatography



MP SP

The stationary phase (**adsorbent**) is chosen to permit differential interaction with the components of the sample to be resolved. The intermolecular forces thought to be primarily responsible for chromatographic adsorption include the following:

- Van der Waals forces
- Electrostatic forces
- Hydrogen bonds
- Hydrophobic interactions

Sites available for interaction with any given substance are heterogeneous. Binding sites with greater affinities, the most active sites, tend to be populated first, so that additional solutes are less firmly bound (**elution**). The net result is that adsorption is a concentration-dependent process, and the **adsorption coefficient** is *not* a constant (in contrast to the partition coefficient).

Separation based on polarity characteristics

Both silica and alumina are **polar adsorbents**, possessing surface hydroxyl groups, and Lewis acid-type interactions determine their adsorption characteristics. The **elution** order of compounds from these adsorptive stationary phases can often be predicted on the basis of their relative polarities. Compounds with the most polar functional groups are retained most strongly on polar adsorbents and, therefore, are **eluted last**. **Nonpolar solutes are eluted first**.

One model proposed to explain the mechanism of liquid-solid chromatography is that **solute** and **solvent** molecules (MP) are competing for active sites on the adsorbent (SP). Thus, as relative adsorption of the mobile phase increases, adsorption of the solute must decrease. Solvents can be rated in order of their strength of adsorption on a particular adsorbent, such as silica. Such a **solvent strength** (or solvent polarity in this case) **scale** is called an **eluotropic series** as shows in **table 8.2**.

Note: If both stationary phase and components in sample are polar, the rate of elute for polar component is slow and hence get separated from rest of sample is passed out of the column last. Therefore, the stationary phase and mobile phase are always opposite. I.e., if the stationary phase is polar, the mobile phase use is non-polar and vice-versa.

Once an adsorbent has been chosen, solvents can be selected from the eluotropic series for that adsorbent. Mobile phase strength (polarity in this case) can be increased (often by admixture of more polar solvents) until elution of the compound(s) of interest has been achieved.

Example: In a sample of a mixture of lipids and proteins. Lipids are non-polar and proteins are polar in nature. Suppose here silica, which is polar in nature, is used as a stationary phase and non-aqueous solvents like **hexane or acetone**, which are non-polar in nature, are used as mobile phase. During separation, lipids come out of the column first (**elution**) while proteins come out later due to their higher adsorption to sand stationary phase. This known as **normal phase chromatography**.

Similarly, if both stationary phase and component in the sample are non-polar, then non-polar component comes out last due to slow rate of elution in the column but remember that a mobile phase must be a polar solvent. This known as **reversed phase chromatography**.

Table 8.2 Eluotropic series of selected solvents

Solvent	Strength (ϵ°)
Hexane	0.00
Chloroform	0.26
Methylene chloride	0.30
Ethyl ether	0.38
Methyl t-butyl ether	0.48
Ethyl acetate	0.48
Acetonitrile	0.52
Tetrahydrofuran	0.53
1- or 2-Propanol	0.60
Methanol	0.70

**Increase
polarity**



Partition (Liquid-Liquid) Chromatography

In partition chromatography, depending on the characteristics of the compounds to be separated, the nature of the two liquid phases can be varied, usually by combination of solvents or pH adjustment of buffers. Often, the more polar of the two liquids is held stationary on the inert support, and the less polar solvent is used to elute the sample components (**normal-phase chromatography**). Reversal of this arrangement, using a **nonpolar stationary phase** and a **polar mobile phase**, has come to be known as **reversed-phase chromatography**.

So, when a sample mixture having components of differences in solubility in different liquids, of same nature, i.e., either polar or non-polar, they get separated into two liquid phases, i.e., mobile phase and stationary phase based on their **partition coefficient** in between two liquids.

Polar **hydrophilic** substances, such as amino acids, carbohydrates, and water-soluble plant pigments, are often separable by **normal-phase** partition chromatography. **Lipophilic** compounds, such as lipids, fat-soluble pigments, and polyphenols, may be resolved better with **reversed-phase** systems. Liquid-liquid partition chromatography has been invaluable to carbohydrate chemistry. Column liquid chromatography on finely divided cellulose has been used extensively in preparative chromatography of sugars and their derivatives. **Figure 8.2** shows a different between **absorption** and **Partition chromatography**

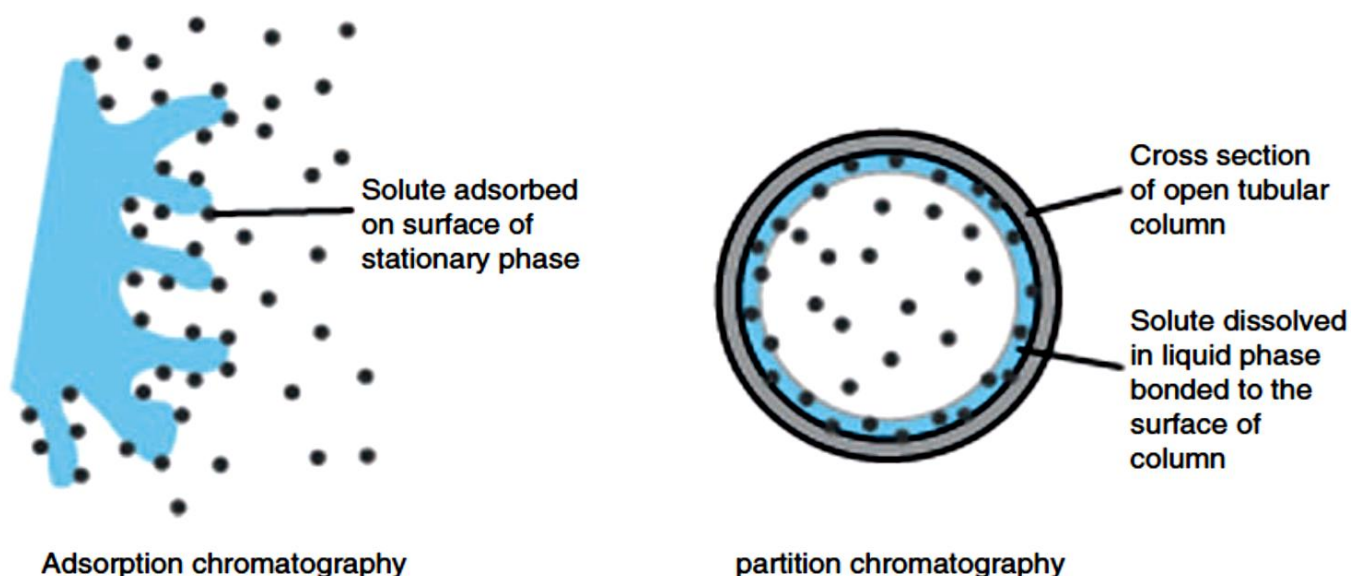


Fig. 8.2 Absorption and Partition chromatography

Paper Chromatography

In paper chromatography the stationary phase (**water**) and the mobile phase (**organic solvent**) are both liquid (**partition chromatography**), with paper (usually cellulose) serving as a support for the liquid stationary phase. The support also may be impregnated with a **nonpolar organic solvent** and developed with water or other polar solvents (**reversed-phase paper chromatography**).

Procedure

The dissolved sample is applied as a small spot or streak about 1.5 cm from the edge of a strip or square of the paper, which is then allowed to dry. The dry strip is suspended in a closed container in which the atmosphere is saturated with the **developing solvent** (mobile phase)

and the paper chromatogram is developed. The end closer to the sample is placed in contact with the solvent, which then travels up or down the paper by capillary action. When the solvent front has traveled the length of the paper, the strip is removed from the developing chamber, and the separated zones are detected by an appropriate method. Paper chromatography is a “**liquid-liquid**” chromatography.

In the case of complex sample mixtures, a **two-dimensional technique** may be used. The sample is spotted in one corner of a square sheet of paper, and one solvent is used to develop the paper in one direction. The chromatogram is then dried, turned 90°, and developed again, using a second solvent of different polarity.

In planar chromatography, components of a mixture are often characterized by their **relative mobility (R_f)** value, where:

$$R_f = \frac{\text{Distance moved by component}}{\text{Distance moved by solvent}} \quad \text{Eq. 8.2}$$

Thin-Layer Chromatography

Thin-layer chromatography (TLC) is a “**solid-liquid adsorption**” chromatography. TLC has largely replaced paper chromatography because it is faster, more sensitive, and more reproducible. The resolution in TLC is greater than in paper chromatography because the particles on the plate are smaller and more regular than paper fibers.

Procedure

In this method, the **stationary phase** is a solid adsorbent substance coated on glass plates. As adsorbent material all solid substances used. In column chromatography (alumina, silica gel, cellulose) can be utilized. In this method, the **mobile phase** travels upward through the stationary phase the solvent travels up the thin plate soaked with the solvent by means of capillary action. During this procedure, it also drives the mixture priorly dropped on the lower parts of the plate with a pipette upwards with different flow rates. Thus, the separation of analytes is achieved. This upward travelling rate depends on the polarity of the material, the solid phase, and of the solvent. **Figure 8.3** shows the components of TLC chromatography.

In cases where molecules of the sample are colorless, fluorescence, radioactivity or a specific chemical substance can be used to produce a visible colored reactive product to identify their positions on the chromatogram. Formation of a visible color can be observed under room light or UV light. The position of each molecule in the mixture can be measured by calculating the relative mobility. Relative mobility value is used for qualitative description of the molecules.

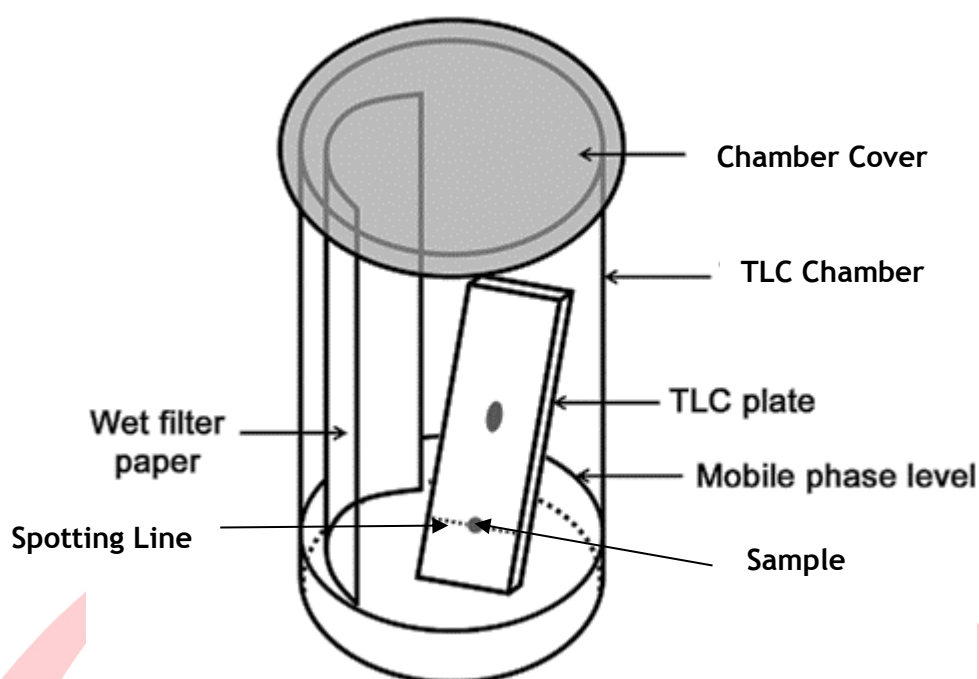


Fig. 8.3 TLC chromatography

High-Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) is a widely used analytical technique that many industries and research fields. This technique yields perfect results in the **separation, identification and quantification** of amino acids, carbohydrates, lipids, nucleic acids, proteins, steroids, and other biologically active molecules.

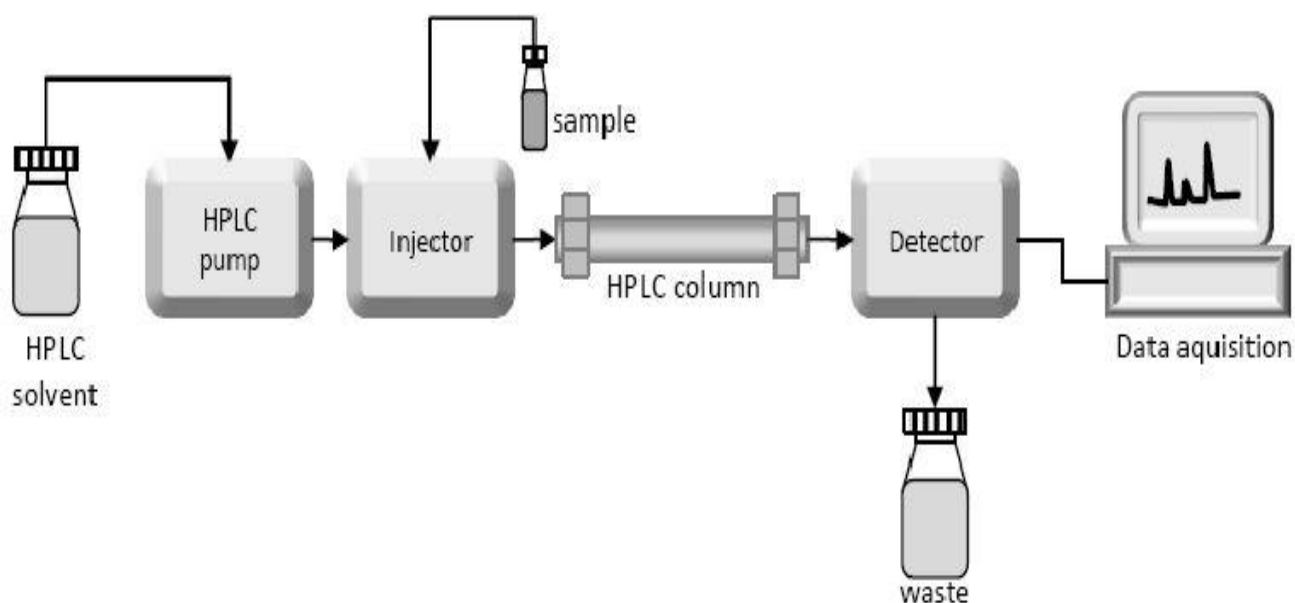


Fig. 8.4 Components of HPLC System

As shown in the schematic diagram in **Figure 8.4**, HPLC instrumentation includes a pump, injector, column, detector and integrator or acquisition and display system. The heart of the system is the column where separation occurs.

1. **Solvent Reservoir:** Mobile phase contents are contained in a glass reservoir. The mobile phase, or solvent, in HPLC is usually a mixture of polar and non-polar liquid components whose respective concentrations are varied depending on the composition of the sample.
2. **Pump:** A pump is used to generate a specified flow of the mobile phase. Depending on a number of factors including column dimensions, particle size of the stationary phase, the flow rate and composition of the mobile phase, operating pressures of up to 6000 psi can be generated.
3. **Sample Injector:** Although manual injection of samples is still possible, most HPLCs are now fully automated and controlled by computer. An injector for an HPLC system should provide injection of the liquid sample within the range of 0.1-100 mL of volume. The injector, or auto sampler, introduces the solvent into a phase stream that carries the sample into the high pressure (up to 400 bar)
4. **Columns:** Columns are usually made of polished stainless steel, are between 50 and 300 mm long and have an internal diameter of between 2 and 5 mm. They are commonly filled with a stationary phase with a particle size of 3-10 μm . Columns with internal diameters of less than 2 mm are often referred to as microbore columns. Ideally, the temperature of the mobile phase and the column should be kept constant during an analysis.
5. **Detector:** The HPLC detector, located at the end of the column. A detector is needed to see the separated compound bands as they elute from the high-pressure column. The information is sent from the detector to a computer, which generates the chromatogram, and to identify and quantitate the concentration of the sample constituents. Commonly used detectors are UV-spectroscopy, fluorescence, mass-spectrometric and electrochemical detectors. For example, if a compound can absorb ultraviolet light, a UV-absorbance detector is used. If the compound fluoresces, a fluorescence detector is used. The mobile phase exits the detector and is either sent to a waste, or collected, as desired.
6. **Data Collection Devices:** Signals from the detector may be collected on chart recorders or electronic integrators that vary in complexity and in their ability to process, store and reprocess chromatographic data. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret. **Figure 8.5** shows in detail the configuration of modules of HPLC

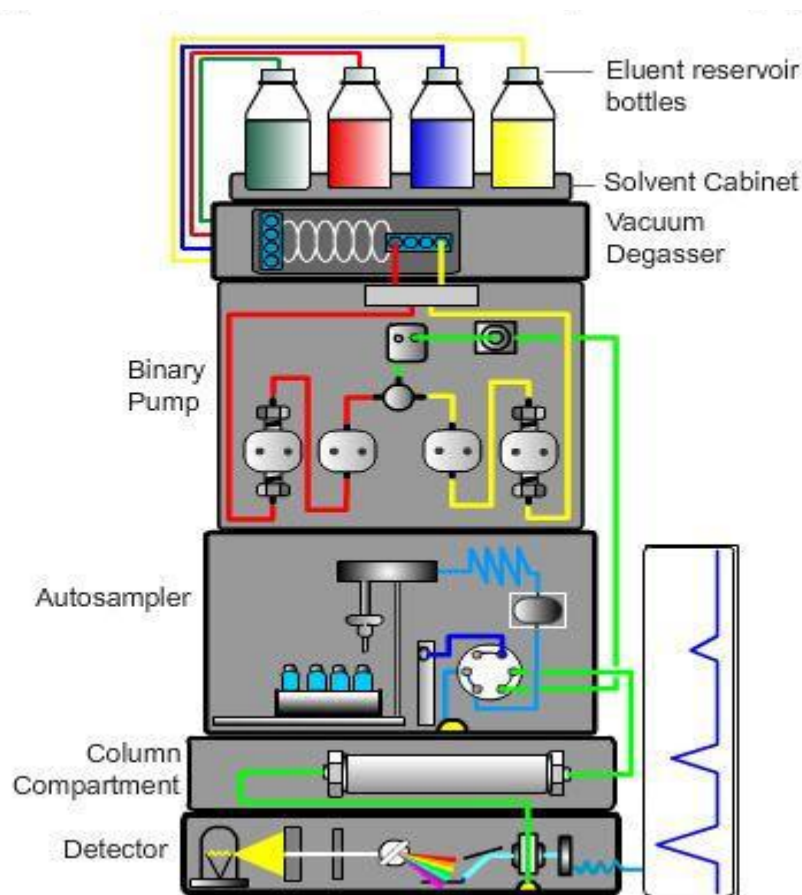


Fig. 8.5 Typical configuration of HPLC

Retention time

The time taken for a particular compound to travel through the column to the detector. This time is measured from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound. In other meaning, is the time, which is needed by a sample component to migrate from column inlet (**sample injection**) to the column end (**detector**).

Different compounds have different retention times. For a particular compound, the retention time will vary depend on:

- The pressure used (because that affects the flow rate of the solvent).
- The nature of the stationary phase (not only what material it is made of, but also particle size).
- The exact composition of the solvent.
- The temperature of the column.

That means that conditions have to be carefully controlled if you are using retention times as a way of identifying compounds.

The volume of the mobile phase required to elute a compound from an LC column is called the **retention volume**, V_R . The associated time is the **retention time**, t_R . Shifts in retention time and changes in peak width greatly influence **chromatographic resolution**.

Differences in column dimensions, loading, temperature, mobile phase flow rate, and detector geometry may lead to discrepancies in retention time. By subtracting the time required for the mobile phase or a non-retained solute (t_m or t_0) to travel through the column to the detector, one obtains an adjusted retention time, t'_R (or volume), as depicted in **Figure 8.6**. The adjusted retention time (or volume) corrects for differences in system dead volume and signifies the time the analyte spends adsorbed on the stationary phase.

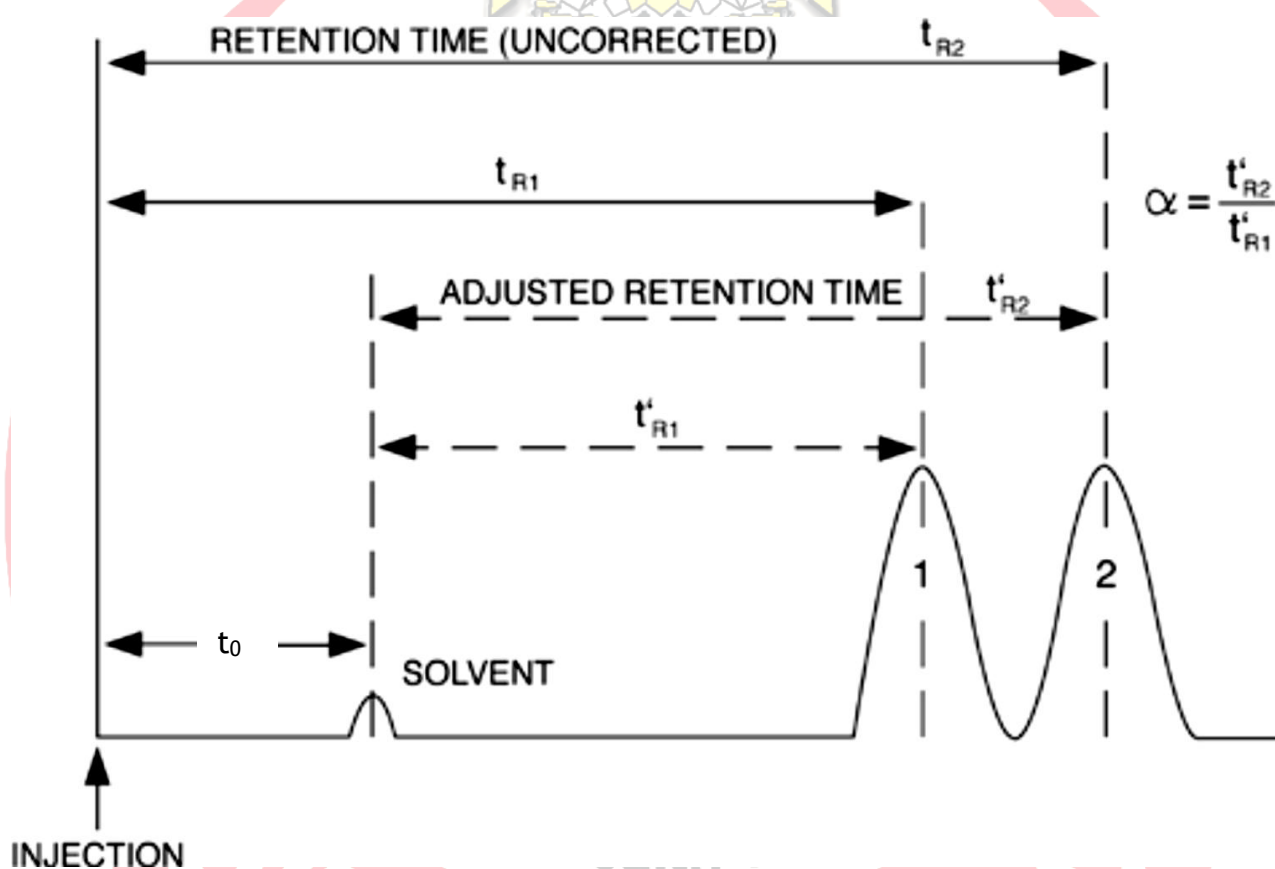


Fig. 8.6 Measurement of chromatographic retention

The **net retention time** (t'_{R1} or t'_{R2}) is the difference between total retention time and dead time. That is the time the sample component remains in the stationary phase.

$$t'_{R1} = t_{R1} - t_0$$

$$t'_{R2} = t_{R2} - t_0$$

Eq. 8.3

The **dead time** t_0 is the time required by an inert compound to migrate from column inlet to column end without any retardation by the stationary phase. Consequently, the dead time is identical with the residence time of the sample compound in the mobile phase.

The **capacity factor** k' is a measure of the position of a sample peak in the chromatogram. It is specific for a given substance. k' depends on the stationary phase, the mobile phase, the temperature, quality of the packing etc.

$$K'_1 = \frac{t_{R1} - t_0}{t_0} \quad k'_2 = \frac{t_{R2} - t_0}{t_0} \quad \text{Eq. 8.4}$$

The **relative retention** α , also known as **separation factor**, is the ratio between two capacity factors, where the figure in the denominator is the reference compound. The relative retention describes the ability of a system of stationary and mobile phase to discriminate between two compounds. It is independent of column construction (length, quality of packing) and flow velocity. It depends on the temperature and the properties of the mobile and stationary phases. Impurities in the mobile phase (e.g. water content) strongly influence the relative retention.

$$\alpha = \frac{k'_2}{k'_1} \quad \text{Eq. 8.5}$$

The **resolution of two peaks** (Figure 8.7) from each other is related to the **separation factor**, α . depend on temperature, flow rate, stationary phase, and mobile phase used. Resolution is defined as follows:

$$R_s = \frac{2 \Delta t}{w_2 + w_1} \quad \text{Eq. 8.6}$$

Where:

R_s = resolution

Δt = difference between retention times of peaks 1 and 2

w_2 = width of peak 2 at baseline

w_1 = width of peak 1 at baseline

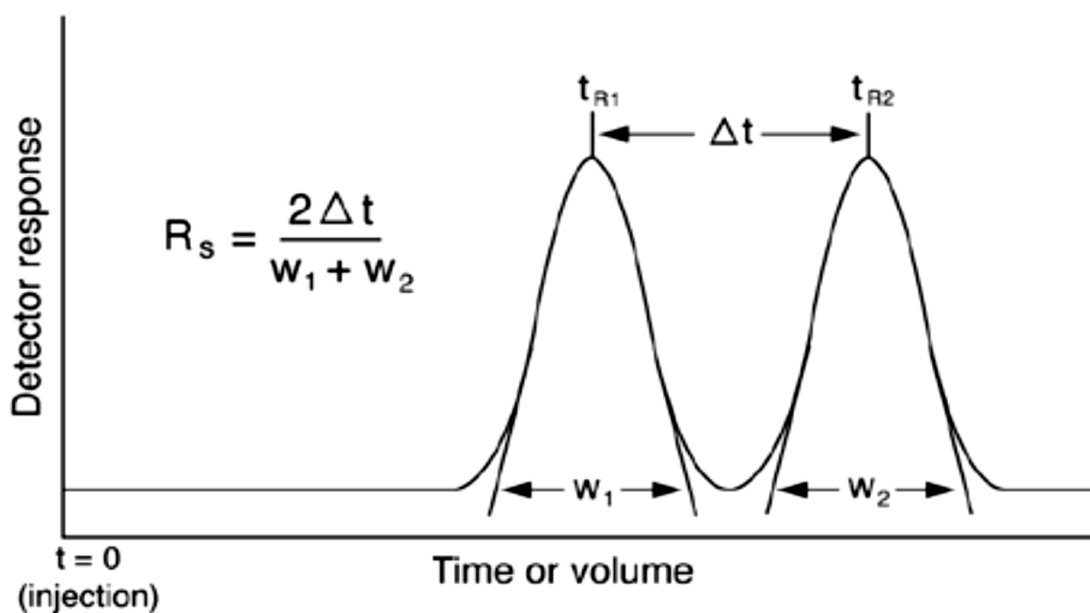
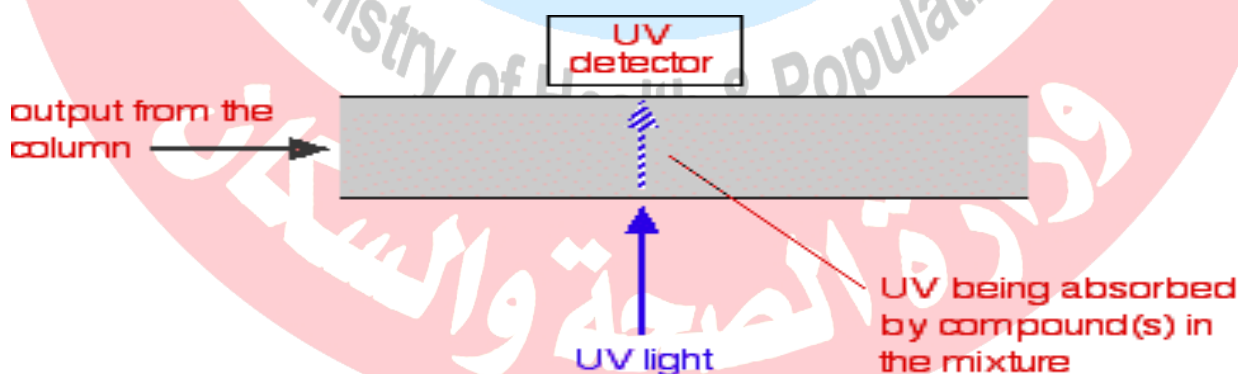


Fig. 8.7 Measurement the resolution of two bands

The detector

There are several ways of detecting when a substance has passed through the column. A common method which is easy to explain uses **ultra-violet absorption**.

Many organic compounds absorb UV light of various wavelengths. If you have a beam of UV light shining through the stream of liquid coming out of the column, and a UV detector on the opposite side of the stream, you can get a direct reading of how much of the light is absorbed. The amount of light absorbed will depend on the amount of a particular compound that is passing through the beam at the time.



Diode array spectrophotometric detectors can provide much more information about sample composition than is possible with monochromatic detection. In this instrument, the light from a **deuterium lamp** is spread into a spectrum across an array of photodiodes mounted on a silicon chip. These are read almost simultaneously by a **microprocessor** to provide the full absorption

spectrum from **200 to 700 nm every 0.1 s**, which may enable the components of a mixture to be identified. Although considerably more expensive than variable-wavelength detectors, they are useful in method development and in routine analyses in which additional evidence of peak identity, without further analysis, is needed.

Fluorescence Detectors

Some organic compounds can reemit a portion of absorbed UV-Vis radiation at a longer wavelength (lower energy). This is known as fluorescence, and measurement of the emitted light provides another useful detection method. Fluorescence detection is both selective and very sensitive, providing up to 1000- fold lower detection limits than for the same compound in absorbance spectrophotometry. Although relatively few compounds are inherently fluorescent, analytes often are converted into fluorescent derivatives. Ideal for trace analysis, fluorescence detection has been used for the determination of various vitamins in foods and supplements, monitoring **aflatoxins** in stored cereal products, and the detection of aromatic hydrocarbons in wastewater.

Refractive Index Detectors

Refractive index (RI) detectors measure change in the RI of the mobile phase due to dissolved analytes, which provides a nearly universal method of detection. However, because a bulk property of the eluent is being measured, RI detectors are less sensitive than other types. Another disadvantage is that they cannot be used with gradient elution, as any change in eluent composition will alter its RI, thereby changing the baseline signal. RI detectors are widely used for analytes that do not contain UV-absorbing chromophores, such as **carbohydrates and lipids**, when the analytes are present at relatively high concentration.

Chromatography is based on the principal that under the same conditions, the time between the injection of a component into the column and the elution of that component is constant. This characteristic is used to perform qualitative or quantitative analysis. Such analyses are explained here using the measurement of aspartame, a synthetic sweetener contained in beverages.

Qualitative analysis

A standard sample of aspartame was measured, yielding a peak at **12.5 minutes**. This peak corresponds to aspartame, itself. Next, a sample prepared from a beverage was measured under the same conditions, yielding a number of peaks. The peak appearing at **12.5 minutes** can be regarded as that of aspartame as shown in **figure 8.8**. The following conditions were the same:

the type of fillers, column sizes, column temperature, composition of the mobile phase, and flow rate.

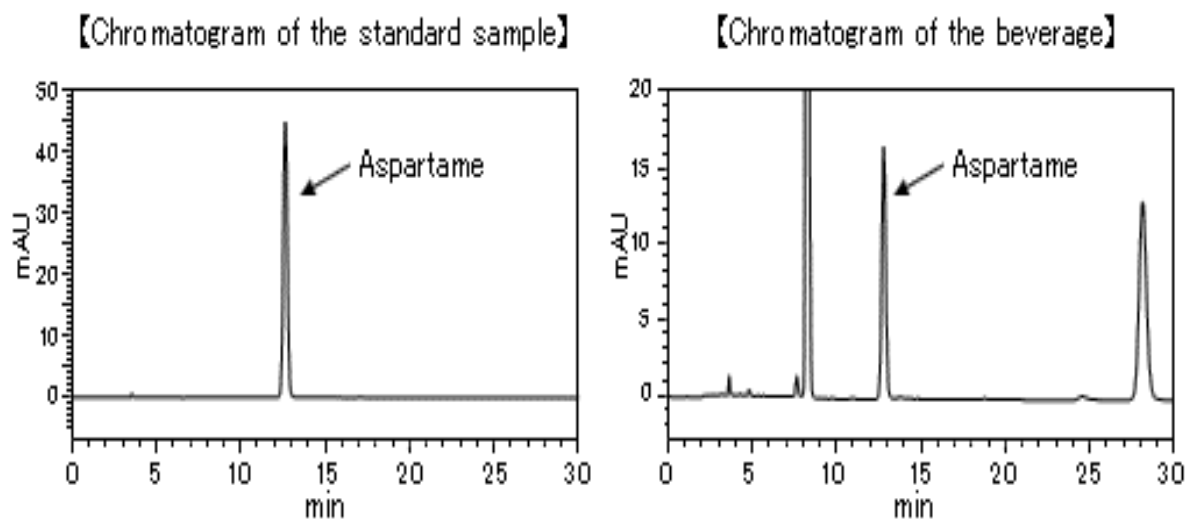
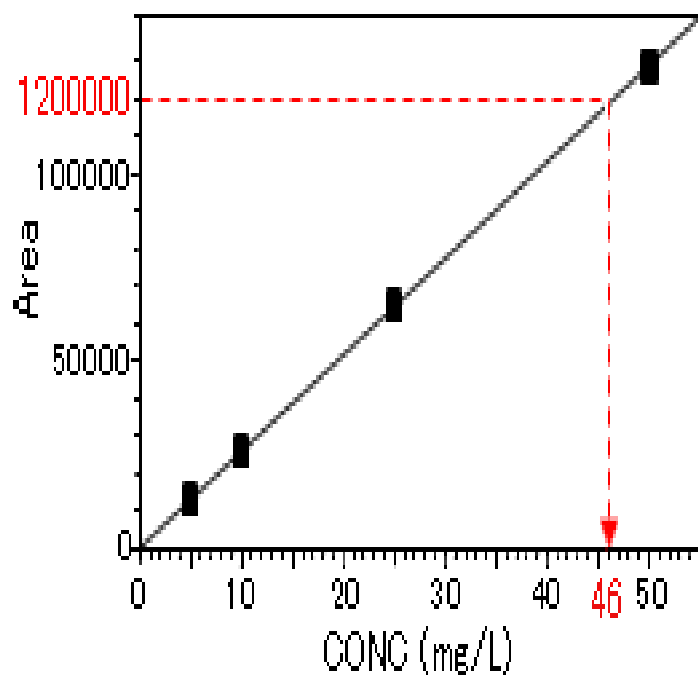


Fig. 8.8 Chromatograph of standard aspartame and beverage sample

Quantitative analysis

The height and area of a peak are proportional to the concentration of the corresponding component. A calibration curve is created using the standard sample. The concentration of aspartame in the beverage can be determined from the peak area of the detected aspartame as shown in figure 8.9.



In the measurement of the real sample, if the peak area of the target component is 120000, the concentration of the component is determined to be 46 mg/L from this calibration curve.

Fig. 8.9 Calculation the concentration of aspartame in beverage sample using HPLC

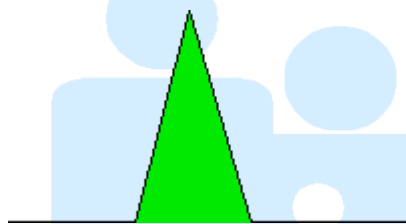
Interpreting the output from the detector

The output will be recorded as a series of peaks - each one representing a compound in the mixture passing through the detector and absorbing UV light. As long as you were careful to control the conditions on the column, you could use the retention times to help to identify the compounds present - provided, of course, that you (or somebody else) had already measured them for pure samples of the various compounds under those identical conditions.

However, you can also use the peaks as a way of measuring the quantities of the compounds present. Let us suppose that you are interested in a particular **compound, X**.

If you injected a solution containing a known amount of pure X into the machine, not only could you record its **retention time**, but you could also relate the **amount of X** to the peak area that was formed.

The area under the peak is proportional to the amount of X, which has passed the detector, and this area can be calculated automatically by the computer linked to the display.

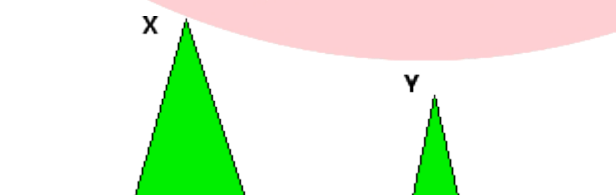


If the solution of X was less concentrated, the area under the peak would be less - although the retention time will still be the same. For example:



This means that it is possible to calibrate the machine so that the resulting **standard curve** can be used to find how much of a substance is present - even in very small quantities.

Be careful, though! If you had two different substances in the mixture (**X and Y**). Could you say anything about their relative amounts? Not, if you were using UV absorption as your detection method.



In the diagram, the area under the peak for Y is less than that for X. That may be because there

is less Y than X, but it could equally well be because Y absorbs UV light at the wavelength you are using less than X does. There might be large quantities of Y present, but if it only absorbed weakly, it would only give a small peak.

Coupling HPLC to a mass spectrometer

Some HPLC machines can be coupled with a mass spectrometer (MS). When the detector is showing a peak, some of what is passing through the detector at that time can be diverted to a mass spectrometer. There it will give a fragmentation pattern, which can be compared against a computer database of known patterns. That means that the molecules can be **identified by the m/z ratio, without the need for knowing the retention times.**

Ultra-High Performance Liquid Chromatography (UHPLC)

Where standard HPLC typically uses column particles with sizes from 3 to 5 μm and pressures of around 400 bar, UHPLC use specially designed columns with particles down to 1.7 μm in size, at pressures in excess of 1000 bar. The main advantage of an UHPLC is **speed**. These systems are faster, more sensitive, and rely on smaller volumes of organic solvents than standard HPLC, resulting in the ability to run more samples in less time. However, if the systems are run at typical pressures greater than 800 bar, the columns age, or degrade quicker. Newer technology is being developed for UHPLC units to use column particles with 1 μm size, and pressure potentials up to 6800 bar. UHPLC is the basis for newly approved **AOAC** official methods for numerous vitamins (i.e., A, B₆, B₁₂, C, D, and folate).



Fig. 8.10 HPLC-MS Instrument

Ion-Exchange Chromatography

Ion-exchange chromatography is based on electrostatic interactions between **charged protein groups**, and solid support material (**matrix**). Matrix has an ion load opposite to that of the protein to be separated, and the affinity of the protein to the column is achieved with ionic ties. Proteins are separated from the column either by changing pH, concentration of ion salts or ionic strength of the buffer solution. Positively charged ion-exchange matrices are called **anion-exchange matrices**, and adsorb negatively charged proteins. While matrices bound with negatively charged groups are known as **cation-exchange matrices**, and adsorb positively charged proteins (**Figure 8.11**).



Fig. 8.11 Type of ion exchanger

The protein of interest is first adsorbed to the ion exchanger under buffer conditions (ionic strength and pH) that maximize the affinity of the protein for the matrix. Contaminating proteins of different charges pass through the exchanger unabsorbed. Proteins bound to the exchangers are selectively eluted from the column by gradually changing the ionic strength or pH of the eluting solution. As the composition of the eluting buffer changes, the charges of the proteins change and their affinity for the ion-exchange matrix is decreased.

Figure 8.12 shows the mechanism of separation, note that the **more highly charged** molecules that are opposite to the ion exchanger are more tightly bound to the exchanger, so they travel slowly and are **eluted later**. In contrast to the **less charged molecules**, which are weakly bonded with the ion exchanger and so travel rapidly and are **eluted in a short time**.

Applications

Food-related applications of ion-exchange chromatography include the separation of amino acids, sugars, alkaloids, and proteins. Many drugs, fatty acids, and organic acids, being ionizable compounds, may be chromatographed in the ion-exchange mode.

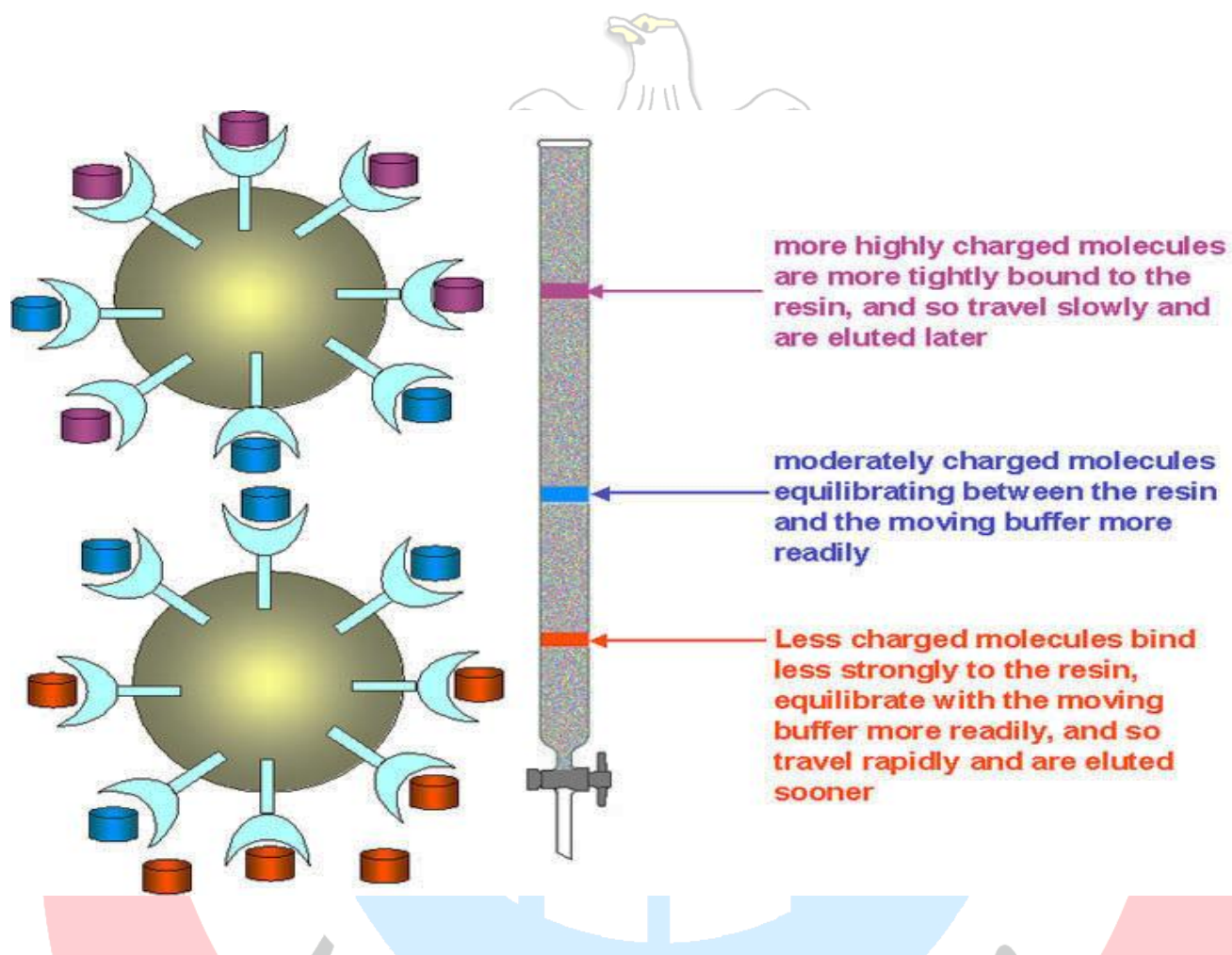


Fig. 8.12 Separation mechanism of ion exchange chromatography

Affinity Chromatography

Affinity chromatography is also another specialized form of adsorption chromatography, with the separation being based on **very specific, a reversible interaction** between a solute molecule and a **ligand immobilized on the chromatographic stationary phase**. Affinity chromatography involves immobilized biological ligands as the stationary phase. These ligands can be antibodies, enzyme inhibitors, lectins, or other molecules that selectively and reversibly bind to complementary analyte molecules in the sample. The principles of affinity chromatography are illustrated in **Figure 8.13**. A ligand which can make a complex with a specific protein (dextran, polyacrylamide, cellulose etc) binds the filling material of the column. The specific protein which makes a complex with the ligand is attached to the solid support (matrix), and retained in the column, while free proteins leave the column. Then the

bound protein leaves the column by means of changing its ionic strength through alteration of pH or addition of a salt solution.

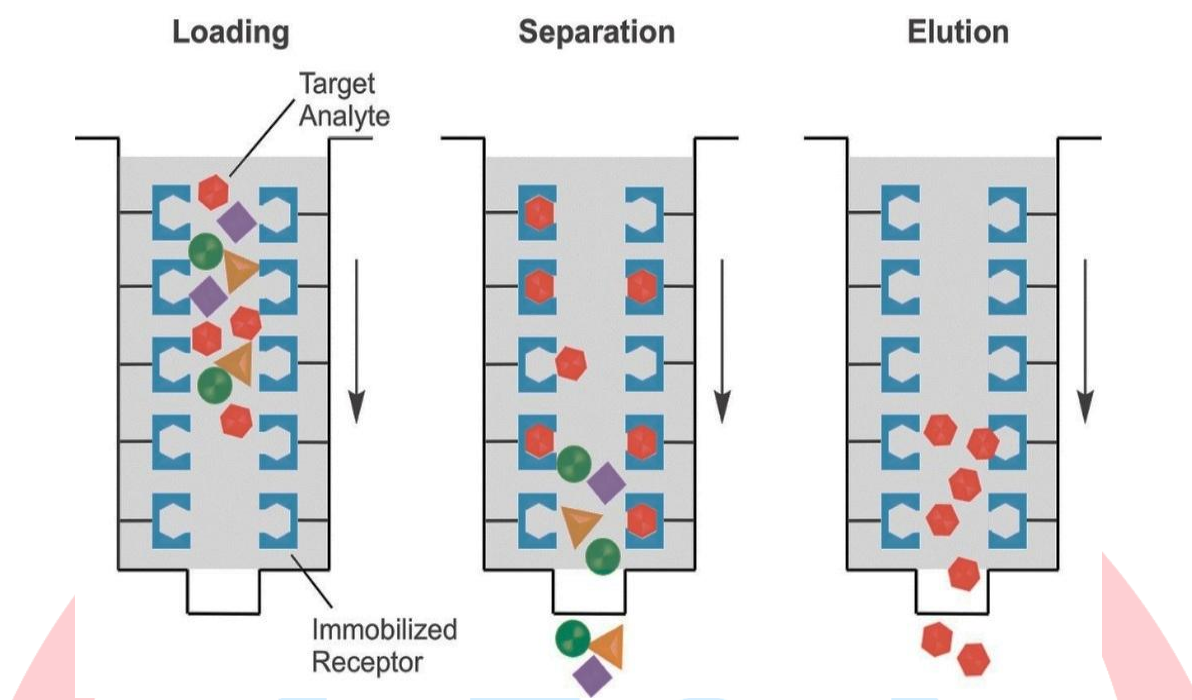


Fig. 8.13 The principles of affinity chromatography

Affinity chromatography is a very powerful technique and is a commonly used protein purification procedure. The average **purification** achieved by affinity chromatography is approximately 100-fold, although 1000-fold increases in purification have been reported.

Ministry of Health & Population

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Size-Exclusion Chromatography

Size-exclusion chromatography (SEC), also known as **molecular exclusion**, **gel permeation (GPC)**, and **gel filtration chromatography (GFC)**. It is widely used in the biological sciences for the resolution of macromolecules, such as proteins and carbohydrates. SEC is a column technique that can be used to separate proteins on the basis of size. A protein solution is allowed to flow down a column packed with a solid support of porous beads made of a cross-linked polymeric material such as **agarose or dextran**. Beads of different average pore sizes that allow for efficient fractionation of proteins of different molecular masses are commercially available.

Molecules larger than the pores in the beads are excluded, moving quickly through the column and eluting from the column in the shortest times. Small molecules enter the pores of the beads and are retarded, thus moving very slowly through the column. Molecules of intermediate sizes partially interact with the porous beads and elute at intermediate times. Consequently, molecules are eluted from the column in order of decreasing size.

Molecular mass can be calculated by chromatographing the unknown protein and several proteins of known molecular mass. Standards of known molecular mass are commercially available and can be used to prepare a **standard curve**. A plot of the **elution volume (V_e)** of each protein vs. **log of the molecular mass** yields a straight line.

Note: Molecules are separated solely on the basis of their size; no interaction occurs between solutes and the stationary phase. The largest molecules are eluted first from an SEC column.

Figure 8.14 shows the mechanism of separation, note that the **large molecules** enter few pores in the gel, and so travel rapidly and are eluted sooner. In contrast to the very small molecules enter many pores in the gel, equilibrating between the gel and the moving buffer, and so travel slowly and are eluted later. A typical Size-Exclusion Chromatogram shows in figure 8.15.

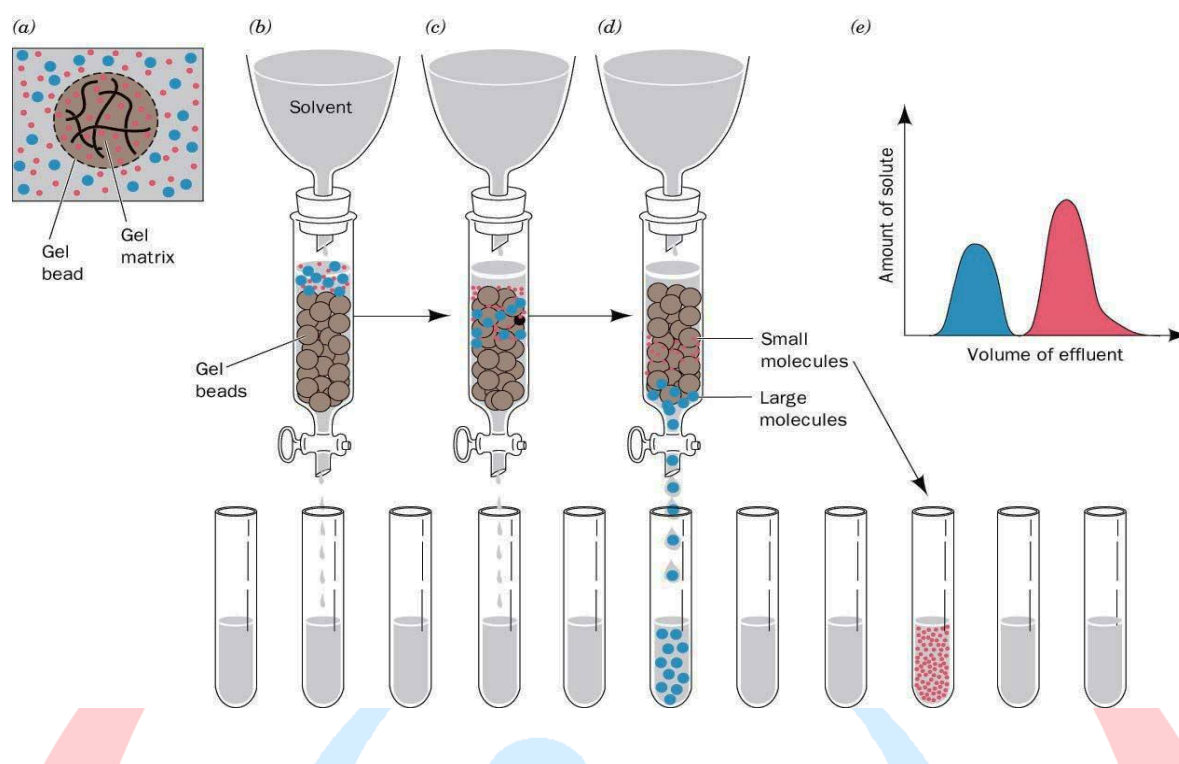


Fig. 8.14 The principles of Size-Exclusion chromatography

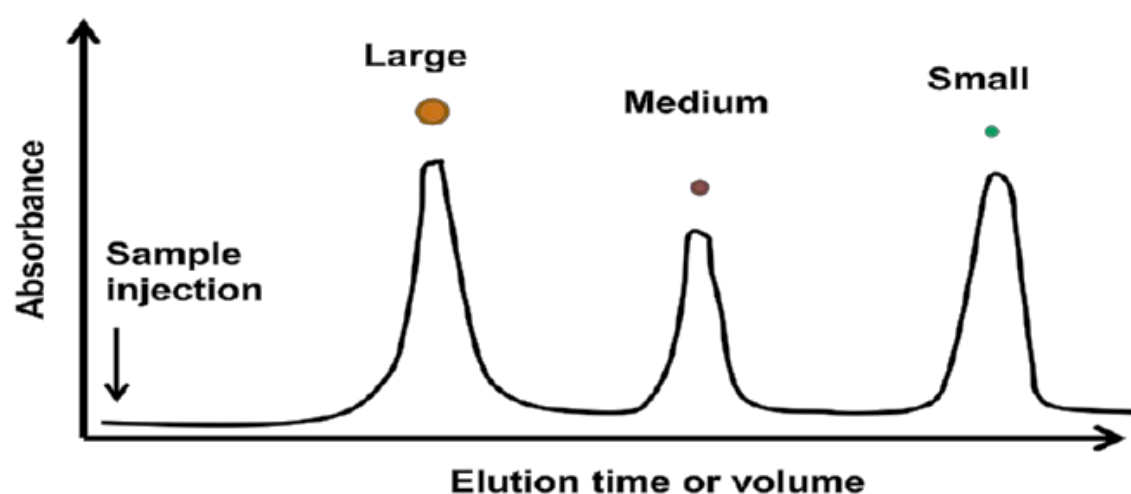


Fig. 8.15 A typical Size-Exclusion Chromatogram

Gas chromatography (GC)

Gas chromatography is a column chromatography technique, in which the mobile phase is gas and the stationary phase is mostly an immobilized liquid on an inert solid support in either a packed or capillary-type column. GC is used to **separate thermally stable volatile components** of a mixture. Gas chromatography, specifically gas-liquid chromatography, involves vaporizing a sample and injecting it onto the head of the column. Under a controlled temperature

gradient, the sample is transported through the column by the flow of an inert, gaseous mobile phase. Volatiles are then separated based on several properties, including boiling point, molecular size, and polarity

Applications

The types of analysis that can be done by GC are very broad. GC has been used for the determination of fatty acids, triglycerides, cholesterol and other sterols, gases, solvent analysis, water, alcohols, and simple sugars, as well as oligosaccharides, amino acids and peptides, vitamins, pesticides, herbicides, food additives, antioxidants, nitrosamines, polychlorinated biphenyls (PCBs), drugs, flavor compounds, and many more.

What different between HPLC and GC?

The two main chromatographic techniques used in modern analytical chemistry are Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC).

HPLC uses a **liquid mobile phase** to transport the sample components (**analytes**) through the column, which is packed with a **solid stationary phase** material.

In contrast, **gas chromatography** uses a **gaseous mobile phase** to transport sample components through either packed columns containing a **polymeric stationary phase**. In most cases, GC columns have smaller internal diameter and are longer than HPLC columns.

Principles

The **carrier gas (MP)** is led at a constant flow rate to the **column packed with the stationary phase**. The mobile-phase gas in GC must be chemically inert and pure. Before entering the column the sample mixture is injected into the carrier gas stream. The sample mixture is vaporized to the gaseous state by the high temperature inside the injector before being led to the column. **The injector is usually heated to 150-250°C which causes the volatile sample solutes to vaporize**. The vaporized solutes are transported into the column by the carrier gas. On reaching the column, the sample components are selectively retained on the basis of physicochemical interactions between the analyte molecules and the stationary phase.

In Gas Chromatography (GC), the mobile phase is a gas and the stationary phase is either a solid "gas solid chromatography (GSC)" or an immobilized polymeric liquid - "Gas Liquid Chromatography (GLC)".

The basic components of a typical instrument for performing GC consists of a supply of carrier gas, a sample injection system, the separation column, the detector, and a data system shows in figure 8.16 .

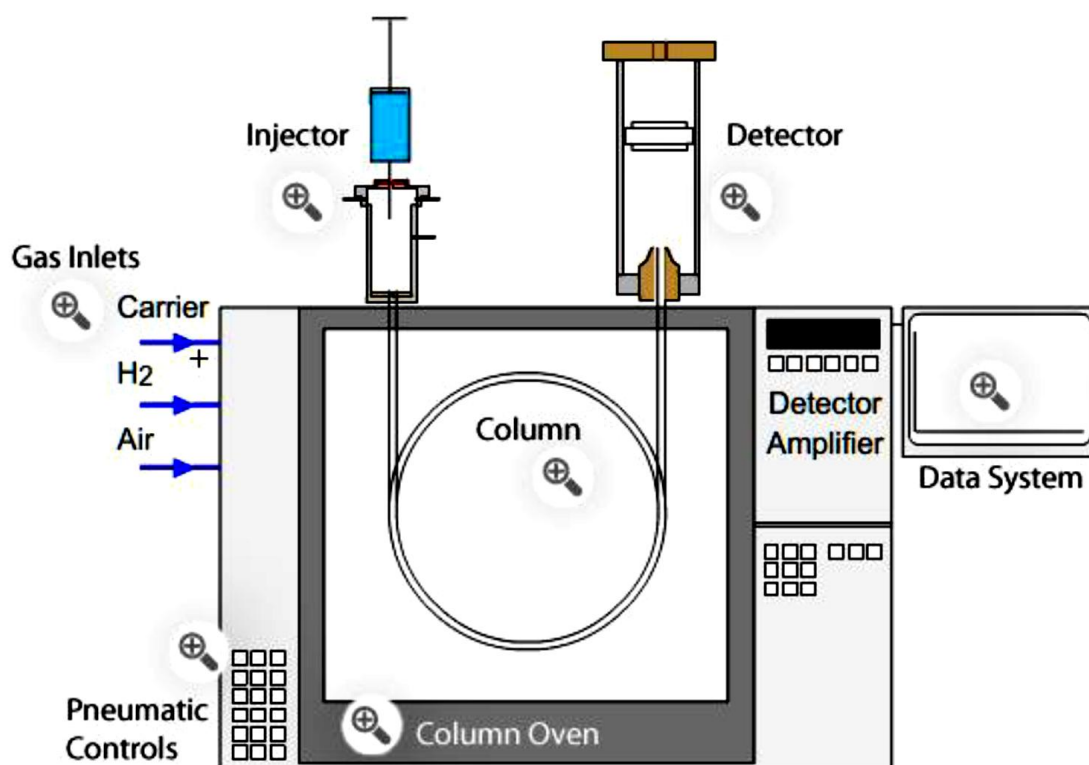


Fig. 8.16 Basic components of GC instrument

Gas Inlets:

Gas is fed from cylinders through supply piping to the instrument. It is usual to **filter gases** to ensure high gas purity and the gas supply may be regulated at the bench to ensure an appropriate supply pressure (Figure 8.17).

Required gases might include:

Carrier - (H₂, He, N₂)

Make-up gas - (H₂, He, N₂)

Detector Fuel Gas - (H₂ & Air, Ar or Ar & CH₄, N₂) depending on the detector type.

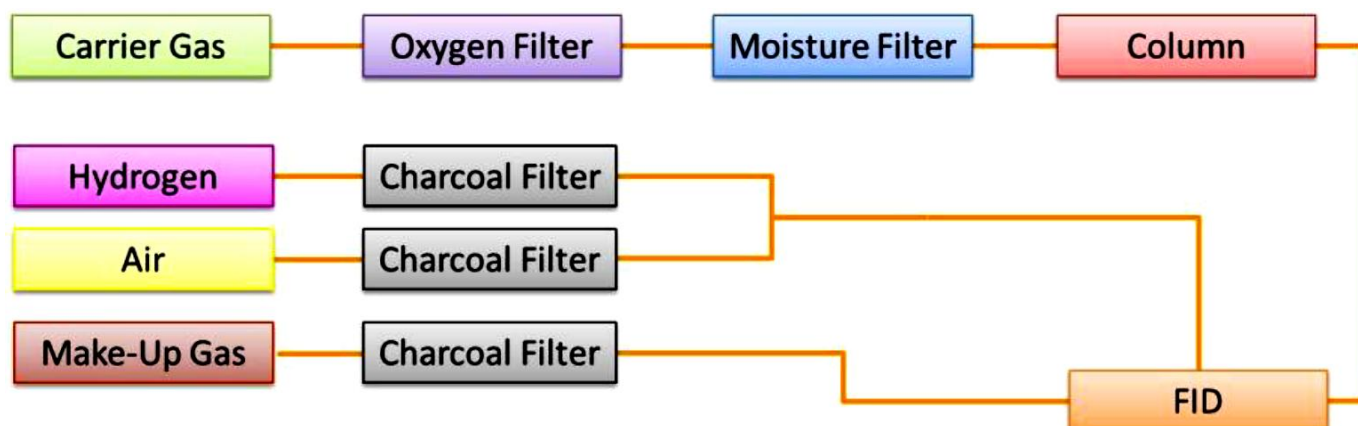


Fig. 8.17 Gas filters required for a GC instrument with Flame Ionization (FID) detector

The gas supply is regulated to the correct pressure (or flow) and then fed to the required part of the instrument. Control is usually required to regulate the gas coming into the instrument and then to supply the various parts of the instrument.

Injection port

An analyte in a very small quantity is injected into the machine through a rubber septum at injection port using a small syringe. Here the sample is volatilized and the resulting gas entrained into the carrier stream entering the GC column.

Column

In GC, retention of analyte molecules occurs due to stronger interactions with the stationary phase than the mobile phase. The sample is separated into its constituent components in the column. Columns vary in length and internal diameter depending on the application type and can be either packed or capillary. Packed columns (typical dimension 1.5 m x 4 mm) are packed with a solid support coated with immobilized liquid stationary phase material (GLC). Capillary columns (typical dimension 30 m x 0.32 mm x 0.1 mm film thickness) are long hollow silica tubes with the inside wall of the column coated with immobilized liquid stationary phase material of various film thickness.

Column Oven:

The column stays inside a thermostatically controlled oven where temperature can be varied between 50-250 °C. This oven does not allow heat transfer from or to the column. The temperature of the injection oven is higher than column oven. The high boiling components can be condensed in the beginning of the column. But as the analysis proceeds the temperature of the column oven rises which can be controlled if necessary.

Detector

The detector responds to a physicochemical property of the analyte, amplifies this response and generates an electronic signal for the data system to produce a chromatogram. Many different detector types exist and the choice is based mainly on application, analyte chemistry and required sensitivity - also on whether quantitative or qualitative data is required.

Detector choices include:

Flame Ionization (FID)

Electron Capture (ECD)

Flame Photometric (FPD)

Nitrogen Phosphorous (NPD)

Thermal Conductivity (TCD)

Mass Spectrometer (MS)

The flame ionization detector is most commonly used in Gas chromatography. In this type of detector organic molecules are burned by flame to get ions and electrons. The positive ions are attracted to cathode to gain electrons and they become neutralized. While the negative ions are attracted to anode to loss electrons electrons. So, The electron flows from electron rich anode to electron deficient cathode through the external circuit. This electric current is more if we have more organic compounds coming out of the column and it is less if there are less compounds. Depending on the amount of current passing through circuit, the computer plots a graph and we can see that on display.

Data System:

The data system receives the analogue signal from the detector and digitizes it to form the record of the chromatographic separation known as the 'Chromatogram' (Figure 8.18). The data system can also be used to perform various quantitative and qualitative operations on the chromatogram - assisting with sample identification and quantitation.

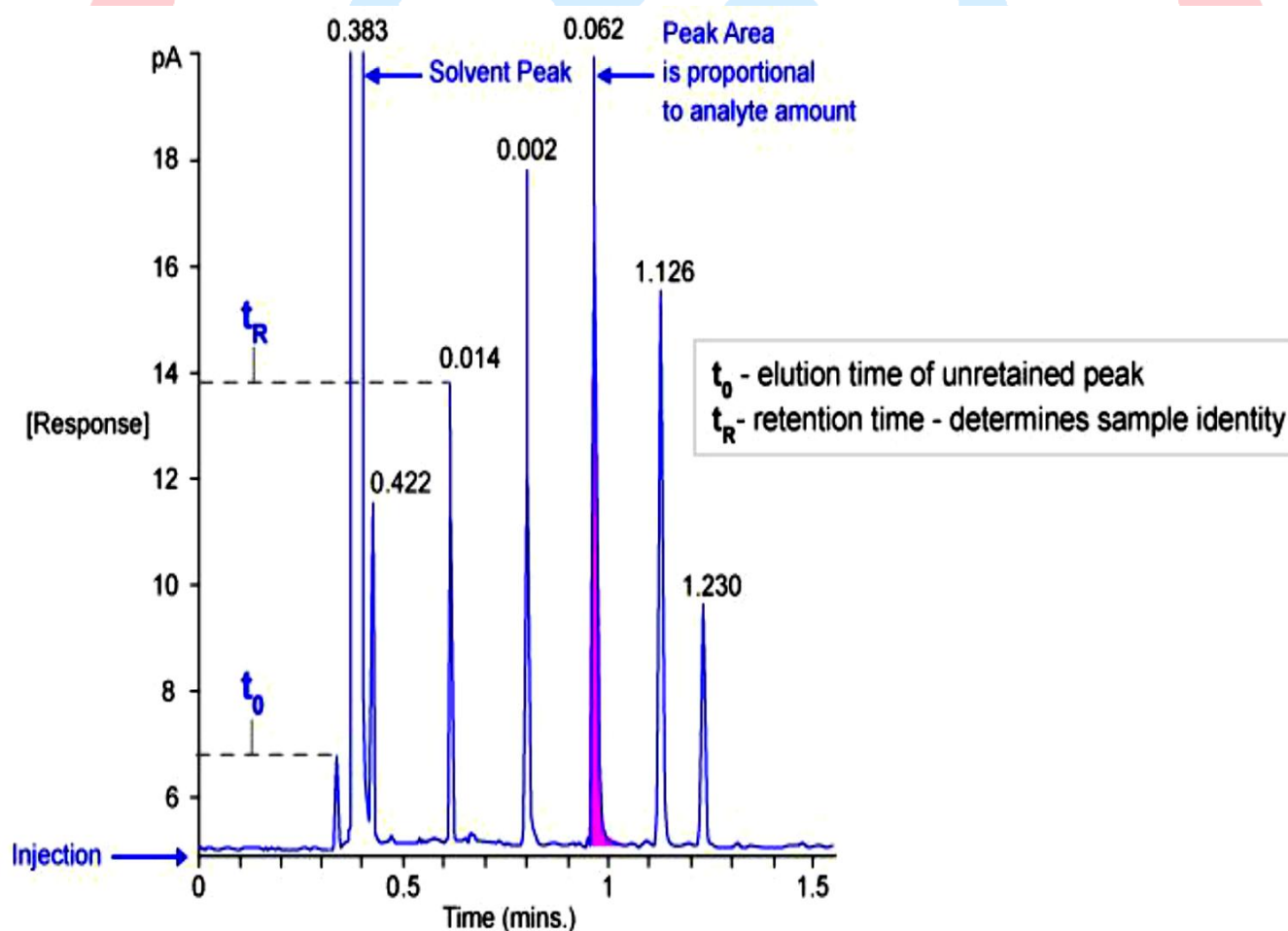


Fig. 8.18 GC chromatogram

The following information gives an indication of the type of sample (analyte) analyzed by either GC and HPLC and relative strengths and limitations of each technique.

Gas Chromatography

- Samples analyzed by GC must be volatile (have a significant **vapor pressure** below 250 °C).
- **Derivatization** to increase volatility is possible but can be cumbersome and introduces possible **quantitative** errors.
- Most GC analytes are under 500 Da Molecular Weight for volatility purposes.
- Highly polar analytes may be less volatile than suspected when dissolved in a polar solvent or in the presence of other polar species due to intermolecular forces such as hydrogen bonding.

High Performance Liquid Chromatography

- HPLC analysis has **no volatility** issues; however the analyte must be **soluble in the mobile phase**.
- HPLC can analyze samples over a wide **polarity** range and is able to analyze ionic samples. Mobile phase components are selected to ensure sample solubility.
- HPLC has no real upper molecular weight limit and large proteins of many thousands of Daltons may be analyzed.

Procedure

- An inert carrier gas, such as helium, is supplied from gas cylinders to the GC where the pressure is regulated using manual or electronic (pneumatic) pressure controls.
- The regulated carrier gas is supplied to the inlet and subsequently flows through the column and into the detector.
- The sample is injected into the (usually) heated injection port where it is volatilized and carried into the column by the carrier gas.
- The sample is separated inside the column - usually a long silica based column with small internal diameter. The sample separates by differential partition of the analytes between the mobile and stationary phases, based on relative vapor pressure and solubility in the immobilized liquid stationary phase.

- On elution from the column, the carrier gas and analytes pass into a detector, which responds to some physicochemical property of the analyte and generates an electronic signal measuring the amount of analyte present.
 - The data system then produces an integrated chromatogram.
 - Gas chromatography uses ovens that are temperature programmable. The temperature of the GC oven typically ranges from 5 °C to 400 °C but can go as low as -25 °C with cryogenic cooling.
- ✓ GC techniques produce fast analyses because of the highly efficient nature of the separations achieved - this will be studied further in the Band Broadening Section. It can be argued that modern GC produces the fastest separations of all chromatographic techniques. A column has been produced to separate 970 components within a reasonable analysis time - proving that very complex separations may be carried out using GC.
- ✓ By using a combination of oven temperature and stationary phase chemistry (polarity) very difficult separations may also be carried out - including separations of chiral and other positional isomers.
- ✓ GC is excellent for quantitative analysis with a range of sensitive and linear detectors to choose from.
- ✓ GC is however limited to the analysis of volatile samples. Some highly polar analytes can be derivatized to impart a degree of volatility, but this process can be difficult and may incur quantitative errors.
- ✓ A practical upper temperature limit for conventional GC columns is around 350-380 °C. Analyte boiling points rarely exceed 400 °C in GC analysis and the upper molecular weight is usually around 500 Da.

GC Advantages and Disadvantages

Advantages

- Fast analysis.
- High efficiency - leading to high resolution.
- Sensitive detectors (ppb).
- Non-destructive - enabling coupling to Mass Spectrometers (MS) - an instrument that measures the masses of individual molecules that have been converted into ions, i.e. molecules that have been electrically charged.

- High quantitative accuracy and precision (<1% RSD typical).
- Requires small samples.
- Separation and analysis of sample very quickly.

Disadvantages

- Limited to volatile samples.
- Not suitable for samples that degrade at elevated temperatures (thermally labile).
- During injection of the gaseous sample proper attention is required.

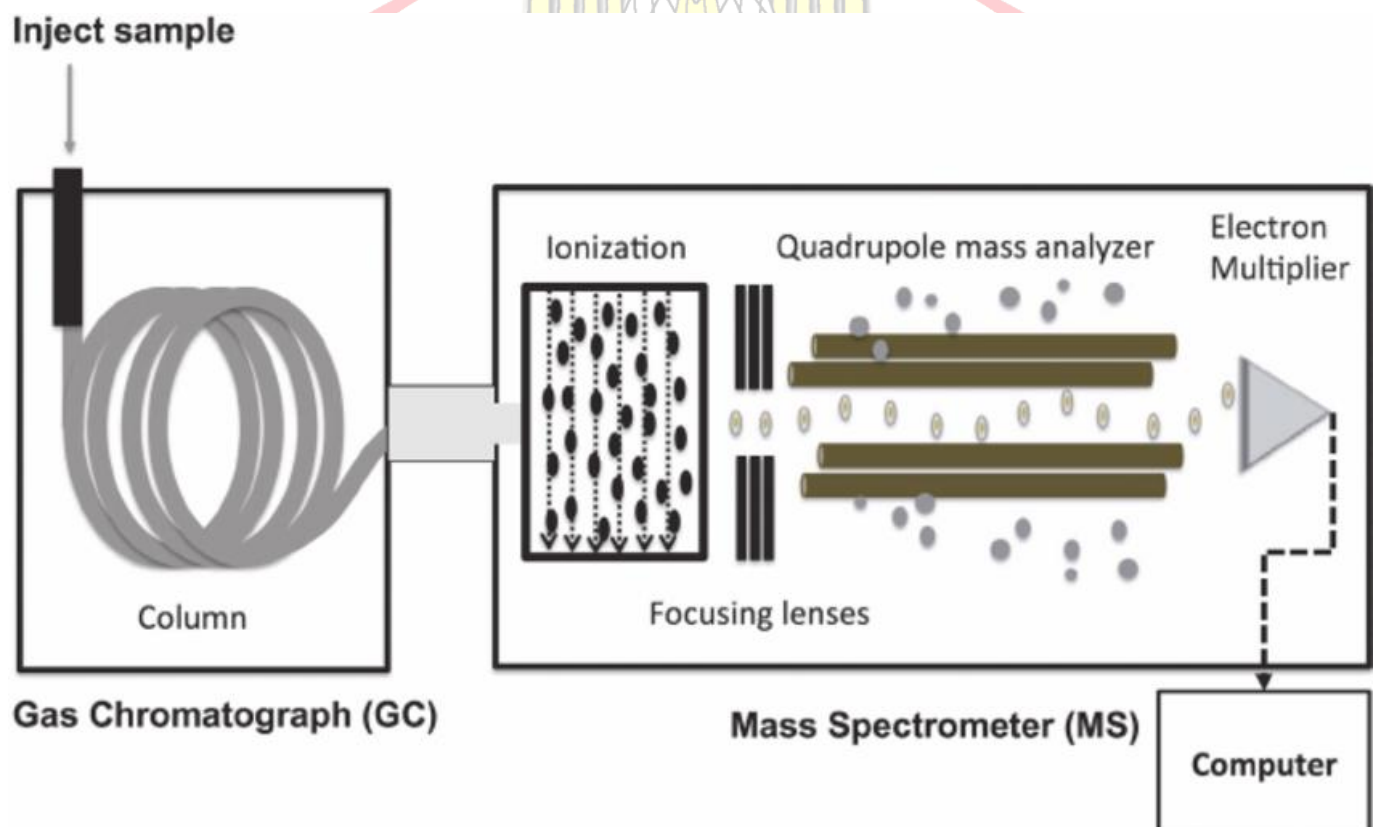


Fig. 8.19 Schematic diagram of a gas chromatography quadrupole mass spectrometer (GC/MS).

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